Identification of Harper-Cawston Factor as Thymidine Phosphorylase and Removal from Media of Substances Interfering with Susceptibility Testing to Sulfonamides and Diaminopyrimidines

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Rich media support the growth of bacteria in the presence of concentrations of sulfonamides and diaminopyrimidines that are highly inhibitory when the organisms are grown on minimal media. Many such rich media can be made more suitable for susceptibility testing by the incorporation of lysed horse blood. Harper and Cawston characterized the active substance, Harper-Cawston factor (HCF), and later studies indicated it to be a protein. It has now been identified as thymidine phosphorylase. The identification follows from the identical purification pattern of HCF and thymidine phosphorylase activities from horse blood to a high degree of purity. Blood of goats, sheep, oxen, geese, chickens, cows, dogs, rats, and humans had neither biological activity. The identification of HCF as thymidine phosphorylase is consistent with the earlier findings of Koch and Burchall (1971) that most of the interfering effects of rich media could be accounted for by their thymidine contents, and that thymidine is much more active in this respect than is thymine.

Since the early work of Colebrook and Francis (4), McCleod et al. (14), and Harper and Cawston (9), lysed horse blood has been used in antibacterial susceptibility test media for reducing the partial growth often observed within the inhibition zones produced by sulfonamides; more recently it has been shown to be similarly effective in susceptibility testing to trimethoprim (2, 5). Harper and Cawston established that (i) lysed horse blood was more effective than whole blood, (ii) bloods of several other species were inactive, (iii) the activity of the lysate increased with incubation time and temperature (up to 30 C), and (iv) the lysate did not affect the reversal of sulfonamide inhibition by *p*-aminobenzoic acid. They concluded that lysed horse blood contained a factor which neutralizes sulfonamide-antagonizing substance(s). More recently, Glaz and Machay (8) partially purified a protein from horse erythrocytes which has the activity of the Harper-Cawston factor (HCF).

In view of the similarity of the problems in susceptibility testing to sulfonamides and to trimethoprim and the beneficial effects of lysed horse blood with both drugs (2, 5), it seemed reasonable that the antagonist in media was an end product of folate metabolism. Such a me-

tabolite could enable organisms to bypass the effects of inhibitors which interfere with either the biosynthesis of dihydrofolate (e.g., sulfonamides) or its reduction (e.g., trimethoprim [7]). Of the probable antagonists, thymidine seemed the most likely, and this was supported by the finding that the inhibitory activity of trimethoprim in a number of commercially available media varied inversely with their thymidine content (10).

The present communication identifies HCF as horse erythrocyte thymidine phosphorylase (EC 2.4.2.4) which cleaves thymidine in growth media to thymine and 2-deoxyribose-1-phosphate. For most organisms, thymine is a poorer blocking agent than is thymidine; thus, the cleavage of thymidine reduces the interference, and growth inhibition by the drugs is restored.

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MATERIALS AND METHODS

Bacterial growth conditions. Wellcotest sensitivity test agar was used in experiments to assess the effects of lysed blood on the reversal by thymine and thymidine of the growth inhibition by trimethoprim

and sulfonamides. This medium was chosen because of its low thymidine content. It was prepared as directed, cooled to approximately 50 C for the addition of blood and other substances, poured into plastic petri dishes, and allowed to stand at room temperature. The plates were seeded with 2 ml of appropriate dilutions of overnight broth cultures of the test organisms, excess fluid was removed, and the surface was allowed to dry at 37 C before the disks containing the antibacterials were applied. In some cases the plates were left 1 h at room temperature to allow for diffusion of the antibacterials. The plates were then incubated at 37 C for 16 to 20 h. The dilutions of inocula were chosen to give a lawn of not quite confluent colonies after overnight incubation. The organisms and dilutions used were: *Staphylococcus aureus* NCTC 6571 (10^{-3}), *Streptococcus faecalis* CN 478 (10^{-3}), and *Escherichia coli* CN 314 (10^{-4}). The zones of inhibition were determined by projection onto a wall chart with sixfold magnification. They are expressed as the distance from the edge of the disk to the edge of growth of uninhibited colonies (clear zones) and to the edge of growth of smaller, partially inhibited colonies, if present (partial zones).

HCF assay. To assay the ability of blood lysates and fractions to increase the antibacterial activity of trimethoprim and sulfonamides on unsatisfactory testing media, a medium was chosen which gave incomplete zones of inhibition. Brain heart infusion agar (45 ml containing 5 μ g of trimethoprim per ml; Difco) was poured into a 150-mm petri dish and, after setting, was seeded with *E. coli* as described above. A steel cork borer was used to cut 6.5-mm diameter wells in the agar, 0.05-ml samples were placed in the wells, and the plate was incubated 18 h at 37 C. At this level of trimethoprim, zones of inhibition could be observed around the wells only if they contained fractions with HCF activity. In each test several dilutions of lysed horse blood were included as standards. Usually the maximal zone of inhibition was obtained with 25% horse blood (zone of 20 mm complete, 25 mm partial), whereas just detectable activity was observed with 3.1% horse blood (zone of 0 complete, 5 mm partial). By this method the HCF content of lysates and fractions could be estimated reproducibly with sufficient sensitivity to follow the purification of the active substance.

Preparation of lysates. HCF activity was demonstrated in horse blood which had been lysed by three different methods.

(i) **Saponin lysis.** Either 2 or 5 ml of sterile 10% (wt/vol) saponin was added to 100 ml of sterile defibrinated horse blood and mixed. The preparation was incubated for 15 min at 37 C, or was left standing at room temperature until the blood was completely lysed, and then stored at 4 C until used.

(ii) **Freeze-dried lysed horse blood.** This product was obtained from Wellcome Reagents Limited as Wellcotest lysed blood (dried) HL03. It is a sterile freeze-dried mixture of whole and heat-lysed defibrinated horse blood which is stable for up to 1 year when stored at 4 C. The powder was reconstituted with sterile water and added to media according to the manufacturer's directions.

(iii) **Osmotic lysis.** Two volumes of cold 0.01 M PO_4 buffer (sodium salts), pH 5.8, were added to packed erythrocytes (plasma and buffy coat removed after centrifugation), and the mixture was shaken vigorously. This method was also used with the other bloods tested.

Thymidine phosphorylase assay. The assay used was based on the higher absorbance of thymine than thymidine at 300 nm in alkali ($\Delta\epsilon_{300} = +3,600$ at pH 12) (7, 12). The standard assay mixture consisted of 0.05 M Sørensen phosphate buffer (pH 7.0), 10 mM thymidine, enzyme, and water to 1.0 ml. Samples of 500 μ liters were removed at 0 time and, after incubation in a 37 C water bath (5 to 30 min), mixed with 500 μ liters of 0.5 N NaOH, and the optical density at 300 nm (OD_{300}) was determined. Control values for thymidine alone and enzyme alone were subtracted from the values for the complete system to calculate the net $+\Delta\text{OD}_{300}$. With lysates it was necessary to deproteinize after incubation by mixing 500 μ liters of sample with 150 μ liters of 2.2 N HClO_4 , and centrifuging 7 min at $40,000 \times g$; 500 μ liters of the supernatant were then added to 500 μ liters of 1 N NaOH, and the OD_{300} was determined. One unit of the enzyme is the amount of protein which catalyzes the cleavage of 1 nmol of thymidine per min. The reaction was linear up to a $\Delta\text{OD}_{300} = 0.200$, and this velocity was only exceeded by the activities of certain individual column fractions.

The synthesis of thymidine from thymine and deoxyribose-1-phosphate was assayed by following the decrease in absorbance at 300 nm. The reaction system consisted of 5.0 mM deoxyribose-1-phosphate, 2.4 mM thymine, and 0.1 M tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride, pH 7.1 (37 C). In one experiment the formation of [^{14}C]thymidine from [$2\text{-}^{14}\text{C}$]thymine was monitored (11). The standard assay was used to follow the cleavage of uridine and deoxyuridine, except that the absorbance was determined at 290 nm (12).

Other methods. All Sephadex gels were swollen for the required length of time in H_2O , washed three to four times in excess starting buffer, and then, after packing into columns, washed with 4 to 5 bed volumes of buffer. The carboxymethyl (CM)-Sephadex used for removal of hemoglobin from horse blood lysates was washed with 0.5 M NaPO_4 , pH 5.8, before the washes with 0.01 M NaPO_4 buffer. Polyacrylamide disc gel electrophoresis was carried out basically according to the procedure of Davis (6) with 10% gels at 3 mA. In several instances, the electrophoresis was continued for a period twice that necessary for the tracking dye to reach the bottom. This was required for adequate separation of the slow-moving protein bands and caused no loss of material, since no bands migrated beyond $R_f = 0.3$. The apparent K_m values for the enzyme were determined from reciprocal plots of $1/v$ versus $1/S$, using the computer program of Cleland (3). The thymidine content of the medium was determined as previously published (10). Proteins were determined by the method of Lowry et al. (13), and hemoglobin was measured as cyanmethemoglobin (Hycel, Inc., Houston, Tex).

Materials. Whole horse blood (defibrinated or

citrated) was obtained from several sources: Wellcome Reagents Limited, Beckenham, England; Granite Diagnostics, Burlington, N.C.; or Robbin Laboratories, Chapel Hill, N.C. Citrated whole bloods of goats, sheep, oxen, chickens, and geese were purchased from Granite Diagnostics. Citrated bloods of humans, cows, rats, and dogs were freshly drawn locally. Wellcotest sensitivity test agar and Wellcotest lysed blood (dried) HLO3 are products of Wellcome Reagents Limited. Thymine, thymidine, and deoxyribose-1-phosphate (cyclohexoammonium salt) were from Sigma Chemical Co.; [2-¹⁴C]thymine (5 mCi/mmol) was from New England Nuclear. The various Sephadex gels were purchased from Pharmacia, diethylaminoethyl cellulose was purchased from Gallard-Schlesinger Chemical Manufacturing Corp., and enzyme grade ammonium sulfate was obtained from General Biochemicals.

RESULTS

The data in Table 1 demonstrate the ability of lysed horse blood to eliminate the interference by thymidine with the inhibitory effects of trimethoprim (T) and trimethoprim-sulfamethoxazole (T+S) on *S. aureus* growth. The addition to the medium of 1.6 μ g of thymidine per ml was sufficient to completely prevent inhibition by the drugs; the inclusion of lysed horse blood (final concentration, 5% [vol/vol]) restored the inhibition. This is also demonstrated in Fig. 1, with *E. coli* as the test

TABLE 1. Effects of thymidine and lysed horse blood on inhibition of *S. aureus* by trimethoprim and trimethoprim-sulfamethoxazole

Expt	Thymidine (μ g/ml)	5% Lysed horse blood	Zone width (mm) ^a	
			T	T + S
1	0	—	9	16
		+	8	14
	0.2	—	(6)	3 (15)
		+	8	14
	1.6	—	0	0
		+	7	15
2	0	—	9	12 (14)
		+	8	14
	10	—	0	0
		+	(8)	(14)
	50, 75, 100	—	0	0
		+	0	0

^a Zone of inhibition measured from the edge of disks of T (1.25 μ g/disk) and T + S (T, 1.25 μ g, + sulfamethoxazole, 23.75 μ g per disk) versus growth of *S. aureus* NCTC6571 on Wellcotest sensitivity testing agar. The values in parentheses are for total zones, including partially inhibited colonies. The values outside parentheses designate the size of zones of complete inhibition only.

FIG. 1. Effects of lysed horse blood and thymidine phosphorylase on the reversal of the activity of trimethoprim and sulfamethoxazole. The upper plate contains Mueller-Hinton medium alone, and the center plate contains the same medium plus 5 μ g of thymidine per ml. The lower plates are similar to the center plate but also contain lysed horse blood (left-hand plate) and thymidine phosphorylase (right-hand plate). The test organism was *E. coli* (CN 314). On each plate the disks contain: upper, 1.25 μ g of trimethoprim plus 23.75 μ g of sulfamethoxazole; lower left, 1.25 μ g of trimethoprim; and lower right, 23.75 μ g of sulfamethoxazole.

organism. The lysed horse blood had no effect on the inhibition observed in this medium in the absence of added thymidine or in the presence of high levels of thymidine (> 25 μ g/ml). For many organisms the inclusion of 10 μ g of thymine per ml had little or no effect on inhibition by the drugs (Table 2). *S. faecalis*, however, is able to use thymine or thymidine, and the inclusion of either at 10^{-6} M completely blocked the inhibitions. For *E. coli* CN 314 it was estimated that thymidine was 30 to 100 times more effective than thymine. This is consistent with published reports on the poor uptake of thymine by wild strains of *E. coli* (15). Similarly, wild strains of *Proteus vulgaris* appear unable to utilize thymine efficiently (16).

Since it appeared that the interfering substance in media was thymidine, it seemed likely that HCF acted by elimination of this folate end product. Thymidine phosphorylase was a likely

TABLE 2. Effect of thymine on the response of a number of bacteria to inhibition by trimethoprim and trimethoprim-sulfamethoxazole on medium with 5% (vol/vol) horse blood

Organism	Thymine ($\mu\text{g/ml}$)	Zone width (mm) ^a	
		T	T + S
<i>Streptococcus faecalis</i>	0	8.0	10.0 (12.0)
CN478	1	0	0
<i>Escherichia coli</i>	0	10.5	11.5
CN314	10	9.6	10.0
<i>Staphylococcus aureus</i>	0	8.0	13.0 (15.0)
NCTC6571	10	8.0	13.0 (15.0)
<i>Haemophilus influenzae</i> CN5893	0	13.0	14.0
	10	10.5	12.5
<i>Klebsiella pneumoniae</i>	0	8.5	10.0
NCTC7242	10	5.0	8.0
<i>Klebsiella aerogenes</i>	0	0	10.0
S2141	10	0	8.0
<i>Proteus mirabilis</i>	0	5.0	9.5
CN2622	10	3.0	7.5
<i>Neisseria gonorrhoeae</i>	0	0	12.0 (15.0)
CN6431	10	0	12.0 (15.5)
<i>Bacillus pertussis</i>	0	0	20.0
CN5756	10	0	20.0

^a Wellcotest sensitivity test agar + 5% whole horse blood (conditions as in Table 1).

candidate since it catalyzes the reaction: thymidine + phosphate $\leftrightarrow$ thymine + deoxyribose-1-phosphate. Accordingly, lysates of horse blood and of the bloods of 11 other animals were assayed for thymidine phosphorylase. Only horse blood was found to have substantial levels of enzyme (80 to 100 nmol/min per ml of lysate; 0.8 to 1.0 nmol/min per mg of Hb). When lysates of the blood cells of humans, oxen, goats, sheep, cows, rats, rabbits, cats, dogs, chickens, and geese were assayed, values of <10 nmol/min per ml of lysate were observed. These lysates were also inactive in the HFC assay; i.e., no zones of inhibition were observed around wells containing these lysates in trimethoprim-containing medium, as described in Materials and Methods.

Lysates of horse blood cells were subjected to several different purification schemes and assayed for HCF and thymidine phosphorylase. Good recoveries (>80%) of both activities were obtained in several preparations from which 95% of the hemoglobin was removed by treatment of lysates with CM-Sephadex gel (fraction 2, Table 3). One such preparation (20 ml, 550 mg of protein) was passed over a Sephadex G-200 column and assayed for thymidine phosphorylase and HCF activity (Fig. 2). Both activities were eluted at the same position, $V_e/V_0 = 1.56$. There was a 71% recovery of

enzymes with a twofold increase in specific activity. When part of this preparation (75 mg) was put on a diethylaminoethyl-cellulose column (0.7 g) equilibrated with 0.01 M phosphate buffer, pH 7.0, it was found that neither the bulk of the protein nor either of the activities was adsorbed. After overnight dialysis against 0.01 M Tris-hydrochloride, pH 8.6 (5 C), the sample was applied to a second diethylaminoethyl-cellulose column in this buffer (Fig. 3). The HCF activity and thymidine phosphorylase eluted together (100% recovery of enzyme; specific activity, 69 U/mg of protein). The preparation at this stage had three major and one minor bands of protein after electrophoresis on polyacrylamide gel. The coincidence of the behaviors of HCF and thymidine phosphorylase during ion-exchange and molecular sieve chromatography strongly argues for their identity. An attempt was made, therefore, to purify the phosphorylase further.

Thymidine phosphorylase purification. All steps in the purification (Table 3) were performed at 4 C. The cells from 175 ml of packed horse blood cells were osmotically lysed, as described in Materials and Methods (fraction 1). The pH was adjusted to pH 6.3 with HCl, and an equal volume of CM-Sephadex gel was added. After stirring for 15 min, the mixture was centrifuged at $23,300 \times g$ for 20 min. The pellet was extracted with 340 ml of 0.01 M pH 5.8 phosphate buffer for 1 h and centrifuged as above, and the resultant pellet was discarded. The supernatant solution from the first CM-Sephadex treatment was mixed with an additional 200 ml of CM-Sephadex gel, stirred, and centrifuged as above, and the pellet was discarded. The combined supernatants (fraction 2) were brought to 45% ammonium sulfate saturation by the addition of solid ammonium sulfate over a 45-min period. After an additional 20 min of stirring, the precipitated proteins were removed by centrifugation (30 min, $23,200 \times g$), dissolved in a minimal volume of 10 mM Tris-hydrochloride, pH 7.9 (5 C), and dialyzed overnight in 200 volumes of the Tris buffer at 7 mM (fraction 3). This material was applied to a QAE-Sephadex column (2.8 by 34 cm) which had been equilibrated with 10 mM Tris-hydrochloride, pH 8.2 (5 C), washed with 35 ml of buffer, and eluted with a gradient of 200 ml of buffer in the mixing vessel and 200 ml of buffer plus 0.5 M NaCl in the reservoir. The enzyme peak eluted at approximately 0.28 M NaCl. The active fractions were pooled, dialyzed overnight in 20 volumes of 2.5 mM Tris-hydrochloride, pH 8.2 (5 C), lyophilized, and dissolved in 7.1 ml of

TABLE 3. Summary of purification of horse erythrocyte thymidine phosphorylase

Fraction no.	Fraction	Vol (ml)	Total units	Protein (mg)	Units/mg of protein
1	Lysate	525	65,559	77,818	0.84
2	CM-Sephadex, batch	940	65,800	3,920	16.7
3	0 to 45% (NH ₄) ₂ SO ₄	44	33,404	884	37.8
4	QAE-Sephadex, concentrated	7.1	19,951	185	108.1
5	Sephadex G-200	46	14,196	77.4	183
6a	CM-Sephadex	13.5	1,051	1.2	880
6b	CM-Sephadex	14.1	1,661	2.25	738

FIG. 2. Chromatography of HCF on Sephadex G-200. A concentrated CM-Sephadex-treated horse blood lysate (20 ml) was applied to a 2.5- by 90-cm column and eluted with 0.01 M phosphate buffer (pH 7.0) (flow rate = 26.4 ml/h). Fractions were monitored for OD₂₈₀ and assayed for thymidine phosphorylase and HCF activities.

H₂O (fraction 4). A 45% loss of activity was observed at the concentration step. This concentrated material was filtered through a Sephadex G-200 column (2.5 by 90 cm) at a flow rate of 9.2 ml/h. The peak of enzyme activity was observed at an elution volume-void volume ratio of 1.66. The pooled active fraction (Fraction 5) was purified over 200-fold but still retained a slight pink color. For a trial run to eliminate the contaminant, approximately 20% of fraction 5 (9 ml) was passed over a CM-Sephadex column (1.5 by 11 cm) in 0.01 M phosphate buffer, pH 5.8, at a flow rate of 0.7 ml/min at room temperature. The enzyme activity was found in one tube (38% recovery, fraction 6a), and the color was retained on the column. The enzyme was concentrated sevenfold by dialysis against solid sucrose and then analyzed by polyacrylamide gel electrophoresis. Only one band of stained protein (R_f 0.1) was observed on a gel containing 8.8 μ g of protein. Three gel electrophoreses (4.4 to 22.0 μ g of protein) were then run for prolonged periods of time to determine if the single band observed could be resolved into

FIG. 3. Chromatography of HCF on a diethylaminoethyl-cellulose column (1.3 by 5.0 cm) in 0.01 M Tris-hydrochloride, pH 8.6 (5 C). The pool of active fractions from the G-200 column (Fig. 2), after passing through a diethylaminoethyl-cellulose column at pH 7.0, was dialyzed against the above buffer and loaded on the column. After washing with 8 ml of buffer, the column was eluted at 1.5 ml/min with a gradient of 100 ml of buffer in the mixing vessel and 100 ml of buffer plus 0.5 M NaCl in the reservoir. The transmission at 280 nm was monitored with an LKB ultraviolet monitor, and fractions were assayed for thymidine phosphorylase and HCF activity.

more than one protein, but single bands were still observed by this procedure. When the remainder (34 ml) of fraction 5 was chromatographed on a similar CM-Sephadex column, gels of the pooled active fractions contained at least two bands of protein. The material was concentrated fourfold by sucrose as described above and rechromatographed on another CM-Sephadex column in 0.01 M phosphate buffer, pH 6.2. The enzyme activity was observed in the first three protein-containing fractions, and the pink color was retained on the column. The pooled tubes (fraction 6b) contained 85% of the enzyme activity applied and showed only one protein band after gel electrophoresis for prolonged time periods (Fig. 4). The

FIG. 4. Tracing of a run of 4.4 μ g of fraction 6b after electrophoresis on a 10% polyacrylamide gel for 75 min at 3 mA followed by 75 min at 6 mA. The gel was stained 4 h with 0.25% Coomassie blue, destained 2 h in 7% acetic acid plus 5% methanol in a Canalcro quick gel destainer, and scanned at 520 nm with a Gilford model 2410S linear transport on a model 240 spectrophotometer.

stability of the enzyme activity in fractions 6a and b were most stable when the solutions were stored frozen, after concentration by sucrose packing.

Enzyme properties. The properties of the horse blood enzyme are similar to those reported for thymidine phosphorylase from other mammalian species (7, 11, 12). When fraction 6b was dialyzed 44 h in three changes of 500 volumes each of 0.01 M Tris-hydrochloride pH 7.6, (5 C) and was assayed by the spectrophotometric method, there was an absolute dependence on phosphate for thymidine cleavage, with a K_m value for phosphate of 3.2×10^{-5} M. The K_m value for thymidine was 3.9×10^{-4} M, and the V_{max} for the pure enzyme was 1,340 nmol/min per mg of protein. The synthesis of thymidine in the presence of thymine and deoxyribose-1-phosphate was observed by spectrophotometric and radioactive assays. The rate was approximately one-half to two times that of thymidine cleavage. Uridine cleavage was not detected in the presence of enzyme from fractions 5 or 6b, but deoxyuridine was cleaved approximately twice as fast as thymidine. The pH optimum in 0.05 M dimethylglutarate- NaPO_4 -NaOH buffer was pH 5.6 to 5.9. The molecular weight was estimated from several Sephadex G-200 columns to be approximately 200,000.

Effects of horse blood thymidine phosphorylase in media. Several additional pieces of evidence support the contention that HCF is thymidine phosphorylase. Plots of enzyme activity versus HCF activity were similar for serial dilutions of preparations at all stages of purity from lysate to 1,000-fold purified enzyme, although the curves were not identical and were somewhat irregular due to the inherent variation of the HCF assay method. The lower limit of HCF activity occurred at enzyme concentra-

tions of about 10 U/ml, when the enzyme preparations were assayed in wells as described in Materials and Methods. Similarly, the bloods of species other than the horse had no HCF activity and contained less than 10 enzyme units/ml. The ability of a crude lysate, a partially purified fraction, and the 1,000-fold purified enzyme to enlarge and clear zones of inhibition on an unsatisfactory susceptibility test medium is shown in Table 4. The activity of the purified enzyme on a thymidine-supplemented medium can be seen in Fig. 1. These data clearly demonstrate that the enzyme can fully duplicate the zone-clearing effects of a horse blood lysate.

The effect of incorporation of a horse blood preparation into an unsatisfactory test medium was explored. In the first experiment a preparation similar to fraction 2 (Table 3) was added to sterile brain heart infusion broth (Difco) to give a final concentration of 2.3 enzyme units/ml, and the broth was incubated at room temperature for 4 h. The thymidine content of the medium, determined by microbiological assay, was 1.15 μ g/ml before the treatment and 0.2 μ g/ml after (82.6% reduction). Agar (1.5%) was then added, the medium was autoclaved, plates were poured and seeded with *E. coli*, and disks containing trimethoprim and trimethoprim plus sulfamethoxazole were added. After overnight incubation at 37 C, zones of inhibition were produced around the T and T + S disks, but they still contained some partially inhibited colonies. The experiment was repeated with a higher enzyme concentration (6 U/ml), higher incubation temperature (37 C), and longer incubation time (18 h). The reduction in thymidine

TABLE 4. Effect of crude and pure thymidine phosphorylase preparations on inhibition of *E. coli* by trimethoprim, sulfamethoxazole, and trimethoprim-sulfamethoxazole

Addition to medium ^a	Enzyme activity (U/ml)	Zones of inhibition (mm) ^b		
		T	T + S	S
None	0 ^c	0 (12)	0 (15)	0 (7)
5% Lysed horse blood	5 ^c	9 (12)	11 (13)	4 (7)
Fraction 2	5.4	10 (13)	12 (15)	6 (9)
Fraction 6b	1.2	10 (13)	9 (15)	6 (9)

^a Brain heart infusion broth (Difco) with 1.5% agar to which additions were made after autoclaving and cooling to 50 to 55 C.

^b Disks of trimethoprim (T, 1.25 μ g), trimethoprim + sulfamethoxazole (T + S, 1.25 + 23.75 μ g), and sulfamethoxazole (S, 23.75 μ g).

^c Estimated.

content was 89.8%, but some colonies that were inhibited only partially were still observed. When sterile lysate or enzyme fractions were combined with the sterile medium at 50 to 55 C, however, clear zones of inhibition were produced (Table 4; Fig. 1). Thus, the pretreatment procedure failed to eliminate thymidine completely and fell short of the effects obtained when active enzyme was present after inoculation and throughout incubation.

DISCUSSION

The early observations of Harper and Cawston (9) on the effects of cell lysis, incubation time, and temperature on the action of horse blood in sensitizing certain media to the antibacterial action of sulfonamides and the demonstration by Glaz and Machay (8) that the active factor is a protein strongly suggested that the HCF is an enzyme in horse blood which inactivates a sulfonamide antagonist in the media. Several studies (8, 9) have eliminated *p*-aminobenzoic acid as the antagonist, a finding confirmed by unpublished observations in these laboratories. The effectiveness of HCF in preventing interference with the inhibitory action of trimethoprim is confirmatory, since trimethoprim acts at a site distal to the utilization of *p*-aminobenzoic acid. It seemed likely that the antagonist which HCF acted upon was thymidine, since Koch and Burchall (10) had reported an inverse correlation between trimethoprim inhibition and thymidine content of various media. Thus, thymidine phosphorylase was a presumptive candidate as the HCF, since (i) the product of its reaction (thymine) is poorly used by many bacteria and (ii) media would provide conditions suitable for its activity.

The following observations demonstrate that HCF is horse blood thymidine phosphorylase. (i) HCF activity and thymidine phosphorylase were both found in horse blood lysates, but not in lysates of bloods of 11 other animals. (ii) Both activities eluted concordantly from ion-exchange and molecular sieve columns. (iii) The ratio of the two activities remained constant (within the limits of the HCF assay) during a purification scheme which resulted in approximately 1,000-fold purification of the enzyme. (iv) The HCF activity of a crude lysate was duplicated by the purest protein fraction, which contained only one band of protein on polyacrylamide gel electrophoresis.

The thymidine phosphorylase of horse blood is most likely derived from the erythrocytes, since removal of the buffy coat did not reduce

the activity. In any case the question which remains unanswered is why horse blood contains high levels of thymidine phosphorylase.

The evidence presented in Table 1 explains the early observations (9) that different amounts of horse blood lysate were required for different media and the fact that it was ineffective in some media. HCF is effective only with media that contain a moderate level of thymidine. At low levels of this metabolite ($<0.1 \mu\text{g/ml}$), the action of the drugs is not antagonized and elimination of this small amount of thymidine has no effect on the inhibition observed. At high levels of thymidine ($>10 \mu\text{g/ml}$), the activity of HCF is not sufficient to overcome the reversal, possibly because not all of the thymidine is cleaved or because the thymidine can be replaced by high concentrations of thymine. Thus, if lysed horse blood is added to media to improve the inhibition by sulfonamides and trimethoprim, it will be effective only in media containing these moderate levels of thymidine. If the zone clearing effect is the sole reason for including horse blood lysate, it would be desirable to choose a medium with moderate to low thymidine content. The incomplete effect of a preincubation with HCF suggests that some agency (phosphatase?) in the medium or microorganism assists in driving the phosphorolysis to completion.

To an extent still to be evaluated, the effects of thymidine phosphorylase depend also on the relative abilities of the bacterial species to utilize thymine in place of thymidine. Exceptionally (e.g., *Streptococcus faecalis*), thymine is well utilized and thymidine-containing media are not markedly improved by HCF.

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