The Protein Synthesis Inhibitor Thermorubin. 2. Mechanism of Inhibition of Initiation on *Escherichia coli* Ribosomes†

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ABSTRACT: The bacteriostatic antibiotic thermorubin (Tr), which forms strong 1:1 complexes with *Escherichia coli* 70S ribosomes ($K_D = 2 \times 10^{-8}$ M) and weaker 1:1 complexes with 30S and 50S subunits ($K_D \sim 2 \times 10^{-6}$ M), inhibits protein synthesis at the level of initiation. Tr inhibition of initiator tRNA binding, as studied by nitrocellulose filter binding techniques, is expressed *only* on 70S particles, and *only* in the presence of initiation factor mixture (IF). Tr does *not* inhibit the formation of 30S·fMet-tRNA·poly(A,U,G)·[GTP,IF] complexes or the subsequent reaction of these complexes and of incomplete 30S complexes with 50S subunits to form normal puromycin-reactive 70S peptidyl-site initiation complexes. In the absence of IF, Tr does *not* inhibit the formation of puromycin-reactive 70S·AcPhe-tRNA·poly(U) "initiation complexes". The latter very likely form directly on 70S particles, not via prior dissociation to subunits. Upon addition of IF, which itself is slightly inhibitory, poly(U)-directed AcPhe-tRNA binding becomes very sensitive to Tr, being 95–100% inhibited at a slight stoichiometric excess of Tr. Poly(A,U,G), a better mimic of the natural "message", absolutely requires the presence of IF for observable binding of either AcPhe-tRNA or fMet-tRNA. Binding of AcPhe-tRNA is then quantitatively inhibited by Tr, whether or not a 20-fold excess of mixed uncharged tRNA is present. Inhibition of fMet-tRNA binding by Tr is nearly 100% when excess tRNA is present but levels off at a plateau of 60% with pure fMet-tRNA alone. Thermorubin acts very early in the initiation process to trap some configuration of a 70S·IF_x complex in the act of opening up to receive poly(A,U,G).

The bacteriostatic antibiotic thermorubin (Tr)[1] inhibits protein synthesis, but not DNA or RNA synthesis, in *Escherichia coli* in vivo (Pirali et al., 1974); in vitro, the binding of fMet-tRNA to ribosomes was partially inhibited, while runoff of initiated polypeptide chains, or poly(U)-directed polyphenylalanine synthesis, continued normally.

The mechanism of initiation on prokaryotic ribosomes [e.g., see Grunberg-Manago & Gros (1977) and Weissbach & Pestka (1977)], with its many controversial unresolved problems, and the specific mechanisms of antibiotic action [e.g., see Vazquez (1979)] are frequently and extensively reviewed and will not be rehearsed here.

We have shown (Lin & Wishnia, 1982) that thermorubin binds quite well to the single sites on both 30S and 50S subunits but binds 100 times more strongly to the combined site on the 70S particle. Here we will show that the binding does not inhibit the subunits but only the intact 70S particle, in a way which differs from the action of other inhibitors of the initiation process.

Materials and Methods

Commercial materials used were the following: Na_2ATP (99%), Na_2GTP (99%), and creatine phosphate (98%) (Sigma); ^3H- and ^{14}C-labeled L-phenylalanine and L-methionine (New England Nuclear); leucovorin (Lederle); puromycin dihydrochloride (ICN); *E. coli* W or B mixed tRNA (Schwarz/Mann and Sigma); *E. coli* $tRNA_f^{Met}$ and $tRNA^{Phe}$ (Sigma); poly(U), [5-^3H]poly(U), and poly(A,U,G) (A:G:U ratio of 1.00:1.61:1.10) (Miles). Buffer A consisted of 100 mM NH_4Cl, 50 mM Tris-HCl, pH 7.5, 7 mM 2-mercaptoethanol, 1 mM GTP, and 5 mM $Mg(OAc)_2$ or as indicated. Buffer B consisted of 50 mM NH_4Cl, 50 mM Hepes, and 5 mM $MgCl_2$, ±0.2 mM GTP, pH 7.5.

Preparations. Ribosomes, subunits, and thermorubin were isolated and/or handled as described previously (Lin & Wishnia, 1982). Crude initiation factor mixture (IF) was prepared essentially according to Traub et al. (1971). $tRNA_f^{Met}$ was charged and formylated by using dialyzed S150 on mixed tRNA or partially purified synthetase/formylase on pure tRNA (Dubnoff & Maitra, 1971; Model & Robertson, 1979). Pure $tRNA_f^{Met}$ accepted 1230 pmol of Met/tRNA A_{260} unit, which is 100 ± 5% of expected values [cf. Weiss et al. (1968)]. The degree of formylation was 84%, by Tris–Cu^{2+} differential hydrolysis (Schofield & Zamecnik, 1968). Pure Ac[^3H]Phe-$tRNA^{Phe}$ was charged as above and acetylated according to Yot et al. (1971).

Binding of Initiator tRNA to 70S and 30S Particles. The nitrocellulose filter technique was used to determine total binding (Nirenberg & Leder, 1964). "Blank" binding of fMet-tRNA to ribosomes was 2–3% of the complete system when poly(A,U,G) was omitted, and 8–10% with IF omitted. Puromycin released more than 90% of the bound radioactivity as pH 5 extractable into ethyl acetate, indicating exclusive

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[1] Abbreviations: S30, 30000*g* supernatant following the initial breakage of *E. coli* cells (essentially total contents less cell wall and membrane debris); S100 or S150, 100000*g* or 150000*g* supernatant of S30 (ribosome free, low in initiation factors, rich in tRNA, elongation factors, aminoacyl-tRNA synthetases, etc.); 30 S, 50 S, and 70 S, small and large ribosomal subunits and the complete ribosome, respectively, signified by their normal sedimentation coefficients; when square brackets do not obviously signify concentrations (e.g., [30S]), they are used to imply that only some of the species listed therein need form part of the specified complex (e.g., 30S·[fMet-tRNA, poly(A,U,G), GTP, IF] implies the *set* of partial and complete 30S initiation complexes containing the bracketed species); ATA, the commercial mixture of compounds called aurintricarboxylic acid; DMF, *N*,*N*'-dimethylformamide; Me_2SO, dimethyl sulfoxide; EtOH, ethanol; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; IF, initiation factor mixture; Tr, thermorubin.

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binding of *N*-acylaminoacyl-tRNA at the peptidyl site (Leder & Bursztyn, 1966).

The final concentration of DMF or Me_2SO, vehicles for adding thermorubin, was kept at 1.0% or less: at 0.4, 1.0, 2.0, and 20% solvent (v/v), the binding of fMet-tRNA relative to the control decreased to 96, 79, 45, and 12% with ethanol; 100, 98, 90, and 18% with DMF; 100, 98, 31, and 20% with Me_2SO. In our hands, poly(Phe) synthesis was also inhibited at low concentrations of DMF.

Figure 1 serves to introduce considerations that we need to recall to the reader at the outset, since they are central to our outlook. We will continually distinguish between equilibrium reactions like the fast formation of 30 S·fMet-tRNA·poly-(A,U,G)·[GTP,IF] [cf. Gualerzi et al. (1977)], where the quantitative consequences of new equilibria (e.g., with thermorubin) can be estimated fairly straightforwardly, and steady-state reactions like the slower GTPase-driven formation of 70 S·fMet-tRNA·poly(A,U,G), where the details of kinetically regulated parallel and sequential recycling are important. In neither case is the observed level of complex formation a direct measure of the "active" fraction of subunits or ribosomes, which in fact we take as unity; however, the quantitative importance of intermediate species remains an open, and vexed, question.

The $[Mg^{2+}]$ dependence of fMet-tRNA binding is well-known [Beaudry et al., 1978; cf. Hershey & Thach (1967)]. The monotonic increase in 30S initiation complex formation and the ascending left branch of mature 70S complex formation (Figure 1) are accounted for straightforwardly from the smooth decrease in the enormous electrostatic repulsion between the polyanionic ribosomal, tRNA, and mRNA reactants with increasing $[Mg^{2+}]$ [cf. Wishnia et al. (1975) and Grunberg-Manago et al. (1981)]. This effect must also somehow produce the descending right branch, in large measure by making it correspondingly harder to pry open some other 70S species at a key step.[2] Direct effects on the final GTPase-driven step are also possible: for the related "coupled" IF-2-dependent GTPase activity of 70S initiation complexes, which also varies with $[Mg^{2+}]$ and $[NH_4^+]$ (Beaudry et al., 1979), one does not yet know how much of the GTP hydrolysis to attribute to the strictly enzymic GTPase of the complete complex and how much to recycling through all or part of the initiation sequence.

Results

Our results are presented in five sections: (A) fMet-tRNA binding to 70S ribosomes [confirmation of Pirali et al. (1974)]; (B) fMet-tRNA binding to 30S subunits (striking new results); (C) effect of uncharged tRNA (interesting mechanistic consequences); (D) binding of AcPhe-tRNA to 70S ribosomes (establishment of the role of initiation factors); (E) poly-(U)-directed poly(Phe) synthesis (further mechanistic consequences).

[2] The effect is not well understood. The actual values of the known decrease in the rate of dissociation of 70S ribosomes into subunits (Wishnia et al., 1975; Chaires et al., 1981) suggest that if this were the bottleneck, the final steady-state levels would not have been reached in 15 min; however, incubation for 1 h does not change the picture (Beaudry et al., 1978). One of us therefore attributed the effect to increasing competition from the uncharged tRNA present (Grunberg-Manago et al., 1981), since the binding of pure fMet-tRNA did show a plateau between 5 and 35 mM Mg^{2+} (when ribosomes were in great excess; Petersen et al., 1976). However, in a study aimed at affinity labeling with AUG derivatives, it was observed that pure fMet-tRNA (added here in considerable excess) showed the typical $[Mg^{2+}]$ maximum (Pongs et al., 1979). The question remains open.

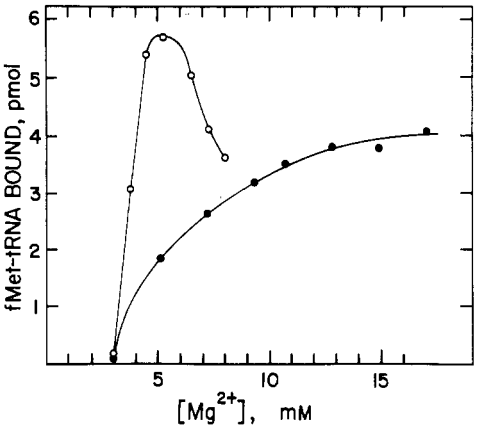

FIGURE 1: $[Mg^{2+}]$ dependence of initiator fMet-tRNA binding to 70S and 30S ribosomal particles. Conditions were the following: final volume 0.1 mL, at buffer A composition, also containing 1 mM GTP, 0.4 mg/mL crude initiation factors (40 μg/tube), 0.8×10^{-7} M f[^{3}H]Met-tRNA (8.0 pmol, 8800 cpm), 2.0×10^{-7} M 70S ribosomes (○) or 4.2×10^{-7} M 30S subunits (●), and $MgCl_2$ as indicated, assembled at 0 °C. Poly(A,U,G), 0.26 A_{260} unit, was added before incubating the mixtures at 37 °C for 15 min (70 S) or 5 min (30 S). The reaction was stopped by adding 2 mL of ice-cold buffer B. Binding of fMet-tRNA to ribosomal particles (i.e., tritium activity retained on the nitrocellulose filters) was measured by scintillation counting of the dried filters in toluene cocktail.

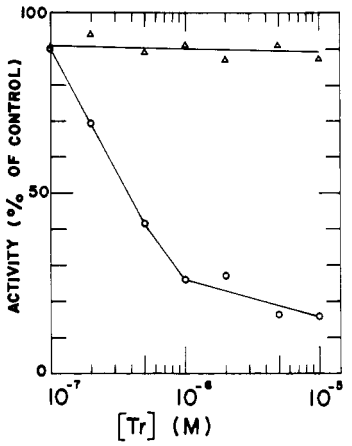

FIGURE 2: Thermorubin inhibition of fMet-tRNA binding to 70S ribosomes. Conditions as in Figure 1, with 5 mM $MgCl_2$, 25 °C. Various dilutions of thermorubin, in constant volumes of DMF–buffer A, were added in two sequences: (A) (○) Thermorubin plus mixture is incubated for 5 min; then poly(A,UG) is added for 15 min of reaction; (B) (△) Mixture plus poly(A,U,G) is allowed to react for 15 min; thermorubin is then added for an additional 5 min of incubation.

(A) Binding of Crude fMet-tRNA to 70S Ribosomes. The effect of thermorubin concentration on this binding is shown in Figure 2. Note that the bulk of the effect (60% inhibition) has occurred at 5×10^{-7} M thermorubin, or a 1:1 Tr:70S ratio [recall that the K_D for 70S·Tr complexes is 2×10^{-8} M [Lin & Wishnia (1982)]]. The semiplateau at about 80% inhibition is typical (see below). All the bound fMet-tRNA, even in inhibited systems, reacts with puromycin to form pH 5 extractable fMet-puromycin (not shown, but see below): i.e., such fMet-tRNA as is bound is bound in the peptidyl site and has normal reactivity. We also observe explicitly what the earlier puromycin reactivity data (Pirali et al., 1974) imply: that thermorubin has no effect when added to preformed mature 70S initiation complexes (triangles in Figure 2).

These results confirm the earlier work (Pirali et al., 1974) and put it on much firmer ground: our system is 30–150 times more efficient at binding fMet-tRNA.

Table 1: Insensitivity to Thermorubin of 30S Preinitiation Complex Formation and the Subsequent Reaction with 50S Subunits[a]

[Tr] ($M \times 10^7$)	10 mM Mg^{2+}, 30 S[b]	5 mM Mg^{2+}, 30 S[c]	5 mM Mg^{2+}, 30 S + 50 S[d]	5 mM Mg^{2+}, 70 S[e]
0	6.90 (100)[f]	ND[g]	7.53 (100)	8.98 (100)
1	6.78 (98)	2.32 (100)	7.06 (94)	7.97 (89)
2	6.63 (96)	2.36 (102)	7.26 (96)	8.21 (92)
4	6.16 (89)	2.24 (97)	7.55 (100)	7.56 (84)
8	6.47 (94)	2.21 (95)	6.80 (90)	4.02 (45)
16	6.83 (99)	2.26 (97)	6.89 (92)	2.30 (26)
32	6.67 (97)	2.22 (96)	ND	2.15 (24)
64	6.41 (93)	2.20 (95)	ND	1.57 (17)

[a] Conditions in Figure 1; thermorubin preincubated 5 min with mixtures as described in Figure 2A. [b] 14×10^{-7} M 30S subunits (15-min reaction, 37 °C). [c] 14×10^{-7} M 30S subunits (5 min, 37 °C). [d] 4.0×10^{-7} M 30S sununits (15 min, 25 °C) plus 5.0×10^{-7} M 50S subunits (5 min more, 25 °C). [e] 11×10^{-7} M 70S ribosomes (15 min, 37 °C). Note again the leveling off above 1 equiv of thermorubin. [f] Values shown are pmol of fMet-tRNA bound (percent of uninhibited equivalent controls). [g] ND, not determined.

(B) Binding of Crude fMet-tRNA to 30S Ribosomal Subunits. Here, the first new, and striking, results were obtained: preincubation with thermorubin had no effect whatever on fMet-tRNA binding to 30S subunits at either 10 mM Mg^{2+}, a common assay choice, or 5 mM Mg^{2+}, the optimum for 70S particles, where discrimination between initiation factor directed and factor-free binding of fMet-tRNA is greatest (Petersen et al., 1976), and which in a functional sense is closer to in vivo conditions [see discussion in Chaires et al. (1981)] (Table I). Note that at the highest [Tr] more than 75% of the noninitiated subunits would be 30 S·Tr.

The question then was whether thermorubin would prevent either the initial binding of the complete preinitiation complex, 30 S·fMet-tRNA·poly(A,U,G)·[GTP,IF], to added 50S subunits, or the subsequent transformation into a mature 70S·fMet-tRNA·poly(A,U,G) initiation complex. Thermorubin had no such effect; indeed, the binding of fMet-tRNA is 2–3-fold higher than for 30S subunits alone and approaches the level expected for uninhibited 70S ribosomes (Table I). Moreover, at least 90% of the fMet-tRNA bound to the 70S complexes (and, of course, none of that bound to 30S complexes) reacted with puromycin, indicating normal placement at the peptidyl site. In other words, all the intermediates smoothly proceed to form mature 70S initiation complexes: once some activation barrier has been crossed, thermorubin has no further effect.

This mode of inhibition of fMet-tRNA binding is not displayed by any other antibiotic [see, e.g., the review by Vazquez (1979)]. Kasugamycin inhibits the formation of both 30S and 70S initiation complexes. Edeine affects mRNA binding to 30S subunits and formation of stable 30S preinitiation complexes (Szer & Kurylo-Borowska, 1972; Jay & Kaempfer, 1975). With pleuromutilin, preincubation in a 30S system before adding 50S particles results in formation of unstable 70S complexes; with preformed stable 70S complexes, the antibiotic inhibits puromycin and peptidyl transfer reactions (Högenauer, 1979).

(C) Effect of Uncharged tRNA. As one step in sorting out the factors responsible for specific 70S inhibition by thermorubin, a study of the binding of pure fMet-tRNA alone and with a 20-fold excess of uncharged tRNA (recall section A above) was undertaken. Typical results are shown in Figure 3. Quite reproducibly, the pure fMet-tRNA system shows a plateau of 60 ± 3% inhibition at thermorubin concentrations above 10^{-6} M, in the given conditions. The inhibition in the

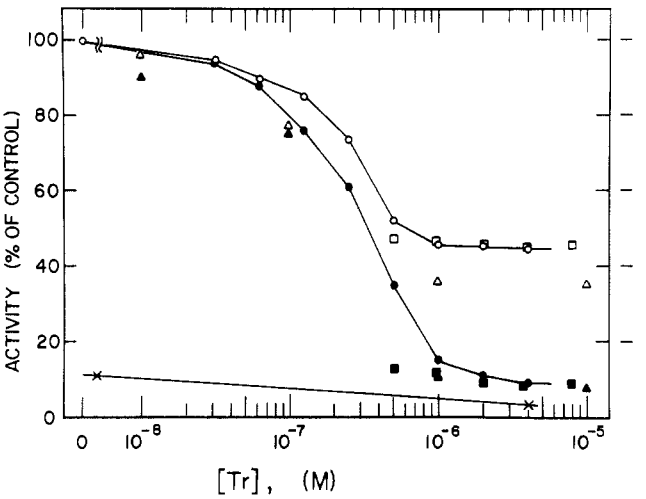

FIGURE 3: Effect of uncharged tRNA on thermorubin inhibition of 70S initiation complex formation. Conditions were the following: buffer B (5 mM Mg^{2+}, 0.2 mM, GTP, etc.), 0.1 mL, containing 25 pmol or 70S ribosomes, 18 μg or crude IF, and 16.7 pmol of pure f[^{14}C]Met-tRNA (450 cpm/pmol); 300 pmol of uncharged, unfractionated *E. coli* tRNA added as indicated; 37 °C. Thermorubin was preincubated with the mixture before adding 0.21 A_{260} unit of poly(A,U,G), as in Figure 2A. Plus (■, ●, ▲) or minus (□, ○, △) uncharged tRNA; (×) plus or minus uncharged tRNA, minus poly(A,U,G). Control values of fMet-tRNA binding (no Tr) were 8.7, 7.0 (■, □); 8.4, 6.8 (●, ○); and 8.1, 6.6 (▲, △) pmol.

presence of excess mixed tRNA is 95–100% complete. The addition of uncharged tRNA alone, without thermorubin, decreases the uninhibited binding from 8.5 ± 0.3 pmol/0.1 mL (0.34 mol of fMet-tRNA/mol of ribosomes) to 6.8 ± 0.2 pmol/0.1 mL.

In general, plateaus are obtained when the binding of an inhibitor (a) modifies steady-state levels by changing one or more rates or (b) exploits or creates two quasi-independent subpopulations. Thiostrepton achieves the latter by inhibiting IF-1-catalyzed recycling of IF-2 (Sarkar et al., 1974): sufficient antibiotic statically traps all the added IF-2 and equivalent amounts of fMet-tRNA in immature, puromycin-unreactive 70S complexes. The plateau observed with streptomycin is attributed to enhanced streptomycin-plus IF-catalyzed decomposition of 70 S·fMet-tRNA·mRNA in the cyclic steady state (Wallace et al., 1979). Neither an intrinsic nor an artifactual two-population model seems appropriate for the thermorubin results: the simple thermorubin binding isotherm (Lin & Wishnia, 1982) and the fact that binding of AcPhe-tRNA is inhibited 100% when the same ribosome and IF preparations are used (section D below) are both inconsistent either with a preexisting distribution (60% sensitive to Tr alone, 40% only to Tr plus excess uncharged tRNA) or with a fractionation by Tr into a totally inert subpopulation (by sequestration of a limiting reagent) and a subpopulation that binds fMet-tRNA very well but is completely inhibited by added tRNA. A dynamic mechanism seems required. Since thermorubin does not destabilize the mature 70S initiation complex, or indeed inhibit its formation from 30S intermediates and added 50S particles, this mechanism must involve attack on a 70S particle at a very early stage (see Discussion). The binding of uncharged tRNA elsewhere might enhance this attack, or tRNA could compete directly for the (modified) P site: tRNA, after all, can bind at both A and P sites, and possibly in several orientations (Watanabe, 1972; Lake, 1977).

(D) Interaction of N-Acetylphenylalanyl-tRNA^{Phe} with Ribosomes. AcPhe-tRNA is often used as an initiator tRNA analogue, although its behavior differs significantly from that of fMet-tRNA: in particular, with poly(U) as "mRNA", GTP

Table II: Poly(U)-Directed AcPhe-tRNA Binding to 70S Ribosomes. Requirement of Initiation Factors for Inhibition by Thermorubin

	A (–IF, –tRNA)		B (–IF, +tRNA)		C (–IF, –tRNA),	D (+IF, –tRNA),
[Tr] (M × 10^7)	binding	PM	binding	PM	binding	binding
0	(5.91)[b]	(5.95)	(4.09)	(4.45)	(3.62)	(1.78)
	100	100	100	100	100	100
0.1	92	101	92	101	98	100
1	93	102	93	105	98	85
2.5	–		–		99	62
10	92	101	97	104	100	26
100	96	103	98	106	101	21
0[a]	4	9	3	8	2	17
100[a]	5	–	3	–	2	16

[a] Blanks without poly(U). [b] The values in parentheses are the actual binding of AcPhe-tRNA in pmol for the controls without thermorubin; the other values are yields as percentages of these. Conditions for B: 37 °C; 5-min preincubation with thermorubin before adding 2 μL (2 μg) of poly(U) to 98 μL of the mixture; 10-min further reaction. A and B contain 25 pmol (2.5 × 10^{-7} M) of 70S ribosomes, 10.8 pmol of AcPhe-tRNA (12 300 cpm ^{3}H/pmol), and *no* GTP. B contains 150 pmol of *E. coli* tRNA. C and D contain 17 pmol of 70S particles, 4.2 pmol of AcPhe-tRNA, and 0.2 mM GTP (plus 27 μg of crude initiation factors in D). Columns labeled PM are yields of AcPhe-puromycin; after reaction, remove 100 μL for 5-min incubation with 1 mM puromycin, quench with 1 mL of 0.1 M NaOAc, pH 5.0, and extract with 1.5 mL of ethyl acetate. All samples counted in Bray's solution.

Table III: Effect of Thermorubin and Initiation Factors on AcPhe-tRNA Binding to 70S Ribosomes. Comparison of Poly(U) and Poly(A,U,G)

[Tr] (M × 10^7)	+poly(U) –IF (+tRNA)	+poly-(A,U,G), –IF	+poly(U), +IF	+poly(A,U,G), +IF
0	3.60[b]	0.07	1.70	0.49
0.1	3.59	0.07	1.57	0.52
1	3.60	0.07	1.05	0.37
10	3.61	0.06	0.14	0.11
100	3.78	0.07	0.15	0.08
0[a]	0.13	0.15	0.17	0.17
100[b]	0.12	0.11	0.11	0.11

[a] Blanks without polynucleotide mRNA. [b] The values shown are pmol of AcPhe-tRNA bound to the filters. Conditions as in Table II, columns A and B [buffer B, 0.2 mM GTP, 25 pmol of 70 S, 10.4 pmol of Ac[^{3}H]Phe-tRNA, 27 μg of IF when present, 2.0 μg of poly(U) or 8.1 μg of poly(A,U,G); 37 °C]. 300 pmol of *E. coli* tRNA present throughout. N.B., compare across as well as down columns.

Table IV: Competition between fMet-tRNA and AcPhe-tRNA for 70S Ribosomes. Effect of Thermorubin[a]

[Tr] (M × 10^7)	fMet-tRNA bound (pmol)	fMet-tRNA bound (pmol)	AcPhe-tRNA bound (pmol)
0	4.96	5.12	0.43
0.1	4.42	4.80	0.40
1	3.27	3.24	0.28
10	0.56	0.54	0.11
100	0.53	0.53	0.10
0[b]	0.63	0.70	0.13
100[b]	0.18	0.18	0.09

[a] Conditions as in Figure 3, Table II (5 mM $MgCl_2$, 0.2 mM GTP, etc). Charge is 2.0 μg of poly(U) plus 8.9 μg of poly(A,U,G), 18 μg of IF, 300 pmol of *E. coli* tRNA, and 25 pmol of 70S particles. Second column, plus 17 pmol of f[^{14}C]Met-tRNA (450 cpm/pmol) alone; third and fourth columns, with 18 pmol of Ac[^{3}H]Phe-tRNA (12 300 cpm/pmol). [b] Blanks without poly(A,U,G)–poly(U) mixture.

and initiation factors are not required. In fact, net AcPhe-tRNA binding in the cyclic steady state driven by GTP hydrolysis, with IF, is substantially lower than that at equilibrium in the absence of IF (cf. Tables II and III); destabilization of at least one configuration of 70 S·AcPhe-tRNA·poly(U) by IF-2 is surely why the complex is a much better coupled IF-2-dependent GTPase than 70 S·fMet-tRNA·poly(A,U,G) [compare Figures 6 and 7 in Beaudry et al. (1979)]. IF-3 catalyzes both formation and decomposition of the 30S initiation complex (Gualerzi et al., 1977; Pon & Gualerzi, 1979).

It was of interest to study the effects of thermorubin, uncharged tRNA, and IF on the AcPhe-tRNA, poly(U), and 70S system. The uninhibited control shows substantial binding, quite comparable with that of fMet-tRNA in standard systems [e.g., 5.9 out of 10.8 pmol of AcPhe-tRNA, with 25 pmol of ribosomes (Table II; cf, Figure 3)]. The inhibition of a 20-fold excess of uncharged tRNA is roughly like that for the fMet-tRNA system (cf. top lines in Tables II and II).

In the absence of crude initiation factors, preincubation with thermorubin before addition of the poly(U) has essentially no inhibitory effect on the binding of AcPhe-tRNA to 70S particles (Table II). The puromycin release data show that AcPhe-tRNA is bound exclusively at the peptidyl site.

Addition of IF mixtures, at amounts optimal for fMet-tRNA binding (cf. Figure 3 and Table IV), produces 50% decreases in AcPhe-tRNA binding with poly(U) as mRNA. Now, however, preincubation with thermorubin produces dramatic inhibition, reducing binding to the background levels of the no-poly(U) blanks, whether excess uncharged tRNA is present or not (Tables II and III). Note, with respect to the two-population alternative discussed in the preceding section, that *no* elevated plateau is observed.

AcPhe-tRNA will also bind at the P site when poly(A,U,G) is the mRNA; there are about as many UUU as AUG (or GUG) triplets in this polymer. Here, binding will not occur *unless* initiation factors are added (Table III). The binding is markedly weaker than with poly(U) (reproducibly, only about 0.4 pmol at comparable conditions; Tables III and IV). It is entirely sensitive to inhibition by thermorubin.

Competition among species was determined in a double-label experiment (Table IV). It is clear that the combination of fMet-tRNA and poly(A,U,G) virtually excludes poly(U), and consequently AcPhe-tRNA binding to poly(U), from the ribosomes [compare poly(U) + IF in Table III with the last column of Table IV]. Total, and therefore also poly(A,U,-G)-directed, AcPhe-tRNA binding is reduced (note that the initial charge of AcPhe-tRNA is almost doubled in the Table IV runs). fMet-tRNA binding is essentially unaffected; the natural initiator is favored by ~15-fold in this system. Thermorubin inhibits the binding of both fMet-tRNA (>90%) and AcPhe-tRNA (~100%) when present in stoichiometric excess.

(*E*) *Poly(U)-Directed Poly(Phe) Synthesis.* Pirali et al. (1974) reported that thermorubin had only a slight effect on poly(Phe) synthesis in an S30 system (total cell contents less

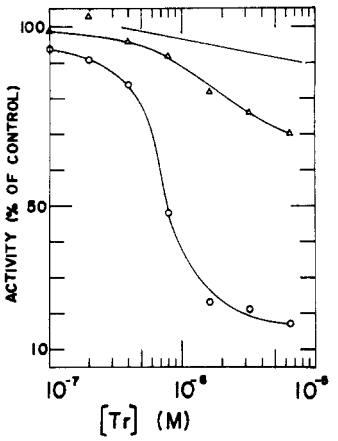

FIGURE 4: Inhibition of poly(U)-dependent poly(Phe) synthesis by thermorubin. Each reaction mixture contained the following components in a volume of 0.125 mL: 100 mM NH_4Cl, 63 mM Tris-HCl (pH 7.8), 18 mM $Mg(OAc)_2$, 0.5 mM EDTA, 16 mM 2-mercaptoethanol, 1.3 mM ATP, 0.3 mM GTP, 13.6 mM creatine phosphate, 8 μg of creatine phosphokinase, 1 mg of *E. coli* wild-type tRNA, 0.04 mM [^{14}C]phenylalanine (10 Ci/mol), 43 μg of S150, 5.3×10^{-7} M 70S ribosomes, 5 μg of poly(U), and various amounts of thermorubin. Mixtures assembled at 0 °C were incubated at 37 °C for 20 min and then quenched with 2 mL of 8% (w/v) Cl_3CCOOH. The mixtures were then heated at 95 °C for 15 min. The precipitate was collected on HAMK Millipore filters and washed 4 times with 2 mL each of 8% Cl_3CCOOH. The dried filters were counted for ^{14}C radioactivity in toluene cocktail. (○) Thermorubin preincubated with partial system for 5 min; poly(U) then added and reaction continued for 20 min. (△) Complete poly(Phe) synthesis system incubated for 5 min; thermorubin then added and reaction continued for 30 min. (—) Inhibition curve from the work of Pirali et al. (1974).

cell wall and membrane debris) which obviously contained substantial amounts of tRNA and the typical fractional complement of IF bound to ribosomes. We investigated both a purified ribosome–elongation factor (EF) system, using preformed Phe-tRNA^{Phe}, with and without excess mixed tRNA and/or IF (Table V, supplementary material), and the system of Wahba & Miller (1971) (Table VI, supplementary material; Figure 4; the S150 fraction used here as the source of Phe-tRNA synthetase and EFs contributes negligible amounts of tRNA or IFs).

Poly(Phe) synthesis is very sensitive to the free Mg^{2+} concentration, increasing 4-fold in a nominal "12", "15", and "18" mM Mg^{2+} sequence (actual, perhaps 8, 10, and 12 mM).[3] The binding of poly(U) to ribosomes, measured indirectly, is also greatly strengthened: aurintricarboxylic acid, at 2×10^{-5} M, inhibits poly(Phe) synthesis by 93% at "12", 52% at "15", and only 15% at "18" mM Mg^{2+} [Table VI; the actual site of ATA inhibition is poly(U) binding (Huang & Grollman, 1972)]. As might be expected from section D, at 8 mM Mg^{2+}, where ATA is most effective, thermorubin does not inhibit directly measured poly(U) binding at all [0 ± 3%, (0–1) × 10^{-5} M Tr; table VI, supplementary material; cf. Smolarsky & Tal (1970)].

We could partially confirm the earlier result, obtained at ~5–6 mM Mg^{2+} (Pirali et al., 1974): at 5–6 mM Mg^{2+}, thermorubin had no effect on poly(Phe) synthesis in the purified system, with or without excess mixed tRNA (Table V); IF markedly decreased the amount of poly(Phe) produced but, at least in one series, did not induce thermorubin sensitivity. However, with both IF and excess tRNA, inhibition reached 60% by 10^{-6} M and 75% by 10^{-5} M Tr (Table VI).

Moreover, at "18" (~12) mM Mg^{2+}, in the absence of IF, preincubation with thermorubin produced a dramatic inhibition, in the usual 10^{-6}–10^{-5} M range (Figure 4). Even addition of thermorubin after 5 min, when more then 30% of the normal synthesis has occurred, produced significant inhibition. The mechanism of IF-free "initiation" (i.e., reactive binding of Phe-tRNA at the peptidyl site) in this system is obscure (recall also the factor-free P-site binding of AcPhe-tRNA), but it is clear that thermorubin certainly prevents such initiation.

Increases in [Mg^{2+}] increase the stability of 70S particles and raise the activation energy for dissociation into subunits (Wishnia et al., 1975). Apparently, at low [Mg^{2+}], thermorubin requires help from other components to clamp 70S particles in an unreactive configuration, while at "18" mM Mg^{2+}, where electrostatic repulsion between 30S and 50S subunits has been minimized, the different ribosome complexes already sit so deeply in their potential wells that binding of thermorubin alone can effectively inhibit the processes of poly(Phe) synthesis.

Discussion

The salient points are that thermorubin inhibits only 70S systems, only when present from the outset, and only in the presence of IF. From this, the inhibited species must contain a 70S particle, one or more components of IF (called IF_x), and thermorubin; i.e., the complex must have the form 70 S·Tr·IF_x·Q. Binding of uncharged tRNA in some (unknown) position clearly potentiates the effect, and so tRNA may form part of Q. The other part may be null: one would like very much to know whether poly(U) or poly(A,U,G) is included. One would like, of course, to know what IF_x consists of.[4]

The question for the data at hand here is which particular function of the IF is operating, to facilitate which structural transformation, that produces a state in which thermorubin binding is inhibitory. As will be seen, a nontrivial conclusion from the "negative" results (i.e., from those systems where thermorubin does not inhibit) is that thermorubin binding and inhibition by thermorubin are not synonymous.

The conventional first, and indeed rate-limiting, step in initiation is the dissociation of ribosomes into 30S and 50S

[3] All the systems described above contain ATP, GTP, and their reservoirs, phosphoenolpyruvate or creatine phosphate, as well as buffers like maleate or acetate, which reduce the free [Mg^{2+}] to levels well below the nominal concentrations [cf. Sillén & Martell (1964, 1971)].

[4] We cannot rule out that IF_x includes the 30S protein S1 or indeed an unknown component of crude IF. There is, of course, voluminous literature on the effect of S1 on the binding of various mRNAs to 30S and 70S particles [e.g., see Steitz et al. (1977), Sobura et al. (1977), Zipori et al. (1978), Draper & von Hippel (1979), and Giorginis & Subramanian (1980)], although as far as we know, no evidence exists that free S1 is present in large excess *in vivo*, where thermorubin is certainly inhibitory (Pirali et al., 1974). As judged from the data for S100 (Van Dieijen et al., 1975), S30 cell sap might contain 0.1–1.0 free S1 per ribosome. Our 70S ribosomes have a reasonable complement of bound S1 [cf. Sobura et al. (1977)]. We can rule out the possibility that thermorubin and IF_x (S1?) sequester one of the nonribosomal components of the initiation complex, *off* the ribosome: for one thing, that would have produced an even greater effect in the 30S system, which is in equilibrium. Moreover, it was observed that augmenting the amount of poly(A,U,G), typically present at slightly suboptimal concentration, by 4-fold, had little effect on the degree of inhibition of fMet-tRNA binding (e.g., 48 → 44% inhibition). Also, by happenstance, binding of AcPhe-tRNA was studied at 3.6, 4.2, 10.8, and 18.2 pmol/0.1 mL, at fixed loads of IF (27 μg) and ribosome (25 pmol): the pattern of inhibition by thermorubin was identical (Tables II and III and data not shown). Hence, neither poly(A,U,G) nor AcPhe-tRNA is sequestered. It is conceivable, one supposes, that thermorubin and IF_x might extract a *ribosomal* component from the 70S particle but not from the separate 30S and 50S subunits. That unlikely prospect is rendered less likely by the fact that thermorubin alone can inhibit poly(U)-directed poly(Phe) synthesis at "18" mM Mg^{2+}, where the ribosome is particularly stable (section E above).

subunits [e.g., as reviewed by Grunberg-Manago & Gros (1977)]. Initiation factors may promote this reaction either by catalysis (if, for example, 70 S·IF$_x$ dissociates faster) or by diverting the products (antiassociation), or both; the role of IF-3 alone is clear, but the crucial effect of mixtures is still not well established [see, e.g., Chaires et al. (1981)]. In this scheme, thermorubin would be inhibitory because 70 S·Tr·IF$_x$·Q is too stable to provide 30S subunits at an adequate rate. Examined in detail, this hypothesis does not yield a satisfactory inhibition plateau (cf. section C under Results).

We have analyzed the kinetics of both antiassociation and prodissociation models, using some simplifying assumptions that would not change the qualitative picture (among them, that all Tr-containing 30S and 50S species react normally in the remaining steps of the stead-state sequence; other tolerable assumptions would make a substantial residual level of fMet-tRNA binding less likely). When the results are scaled to the uninhibited rates for the same model, one obtains relative steady-state levels of fMet-tRNA binding less than or equal to $(1 + K_{A,30}[Tr])([+ K_{A,50}[Tr])/(1 + K_{A,70}[Tr])$ (antiassociation, Appendix II; see paragraph at end of paper regarding supplementary material) or $K'_{A,30}/K'_{A,70}$ [prodissocation, cf. Appendix I (supplementary material)], where K_A and K'_A are association constants for binding thermorubin to free 30S, 50S, or 70S particles (5×10^5, 5×10^5, and 5×10^7 M^{-1}; Lin & Wishnia, 1982) or to 30 S·IF$_x$ and 70 S·IF$_x$, respectively. In the first case, the residual fMet-tRNA binding would not exceed 5–7% at 10^{-6}–10^{-5} M Tr, far from the desired 40%. The K'_A values are not known, but since $K'_{A,70}$ is no less than 5×10^6 M^{-1} (half-maximal effect in Figure 3), and since, again, inhibition is complete in 70S·AcPhe-tRNA systems and nil in 30S systems, it is unlikely that $K'_{A,30}/K'_{A,70}$ is anywhere near 0.40.

The analysis is too indirect for all its consequences to be accepted without other confirmation, but the clear implication is that in Tr-inhibited systems either the 30S·[Tr,IF,Q?] species retains the memory of the act of dissociation in its configuration, and differs from the species present when one begins with 30S particles, or, more plausibly, the entire process occurs on the 70S·Tr particle. In either case, the modified reactivity of an intermediate beyond the conventional first step generates the new steady-state levels.

Let us consider, in this light, the equilibrium cases, where GTP hydrolysis is not involved and where thermorubin does not inhibit: 30 S·fMet-tRNA·poly(A,U,G)·[GTP,IF] and 70 S·AcPhe-tRNA·poly(U). At 10^{-6}–10^{-5} M Tr, 70 S·Tr predominates over free 70 S by factors of 50–500; the fraction [30 S·Tr]/[30 S + 30 S·Tr] shifts from 0.3 to 0.8. How can addition of thermorubin *not* displace the observed equilibrium amount of fMet-tRNA bound to 30S subunits or the amount of AcPhe-tRNA bound in the peptidyl site of 70S complexes?

(1) The obvious way is if the thermorubin binding site and the various 30S·fMet-tRNA·poly(A,U,G)·[GTP,IF] and 70S·AcPhe-tRNA·poly(U) binding sites do not interact strongly with each other (i.e., if the binding of thermorubin does not significantly change the free energy of binding of the initiation components in these systems).

(2) The alternative is that binding of thermorubin and of initiation components is indeed mutually exclusive, but the following rather special conditions are met: (a) either the ratio [70 S·Tr]/[70 S free] shifts with [Tr] without changing the sum of [70 S·Tr] and [70 S free] (likewise for 30 S) or (b) the concentration of free 70S particles in the initiation mixtures is already so low that the 500-fold increase in the sum [70 S free] + [70 S·Tr] at 10^{-5} M thermorubin is still negligible. Case 2a requires that the degree of formation of initiation complexes be determined by a limiting reagent whose binding to ribosomal particles is so strong as to be quantitative, thereby creating two independent populations; the shift from free 70 S to 70 S·Tr in the fixed second population would then have no observable effect. Case 2b requires that the concentrations and formation constants of all the initiation intermediates [e.g., 70 S·poly(U)] be so large that [70 S free] is infinitesimal; complete and partial complexes predominate overwhelmingly.

Case 1 may sound improbable, but cases 2a and 2b are, in our experiments, *impossible*. For the same 70S, poly(U), AcPhe-tRNA system, neither of the two candidates, AcPhe-tRNA or poly(U), is either quantitatively (2a) or overwhelmingly (2b) bound (cf., e.g., Table II or the analysis under Results, sections D, E). For fMet-tRNA and 30S subunits, one notes the increase in binding with increasing [Mg^{2+}] and the still greater increase when 50S subunits are added (Figure 1, Table I). The evidence available insists that thermorubin binds to the various 30S·[fMet-tRNA, poly(A,U,G), IF] and 70S·[AcPhe-tRNA, poly(U)] species in a way which does not greatly modify the overall concentration of these complexes.

Let us now consider the kinetic behavior in the 30S system and the AcPhe-tRNA, poly(U), 70S system. When 50S subunits are added to a 30S subunit initiation reaction mixture containing thermorubin, the equilibria can in principle be displaced in two directions: (a) by the normal formation of 70 S·fMet-tRNA·poly(A,U,G)·[GTP,IF] [see, e.g., Sarkar et al. (1974)] followed by the translocation-like formation of the P-site complex via GTP hydrolysis and loss of IF; (b) by association of free 30S and 50S subunits into 70S ribosomes and sequestration as 70S·Tr and 70S·Tr·IF$_x$·Q complexes. The first reaction in (b) would take less than 1 s at our concentrations (Wishnia et al., 1975); even the presence of 30S·IF-3 complexes would not raise the apparent half-time of formation of 70S particles to more than ~20–30 s (Chaires et al., 1981). Nevertheless, the path taken is clearly (a): bound thermorubin puts no new activation barriers in the complex path of macromolecular conformation changes and shunting, involving the subunits, poly(A,U,G), IF, and fMet-tRNA, needed to carry out the processes.

Analysis of the 70S, AcPhe-tRNA, poly(U) system is also instructive. Formation of the complex requires no IF, but without IF, the putative 70S → 30S + 50S bottleneck would contribute at least 6 min to the half-time for the overall reaction; when dissociation can occur only via 70 S·Tr ⇌ 30 S·[Tr] + 50 S·[Tr] and 70 S·Tr + Tr ⇌ 30 S·Tr + 50 S·Tr, the half-time would be 10–100 times longer (Appendix I). Yet, when poly(U) is added to a mixture of ribosomes, thermorubin, and AcPhe-tRNA, *no* inhibition of formation of 70 S·AcPhe-tRNA·poly(U) is observed. It is difficult to avoid the conclusion that poly(U) and AcPhe-tRNA bind directly to 70S particles without ever passing through a stage of 30S intermediates. Poly(U), of course, has long been known to bind functionally to ribosomes at Mg^{2+} concentrations where even transient dissociation into subunits is unthinkable [cf. Wishnia et al. (1975) and Chaires et al. (1981)], for example, at 8 mM Mg^{2+} (Table VII) and ~12 mM Mg^{2+} (Figure 4). What is clear here, however, is that there are no serious activation barriers, requiring catalytic intervention by IF, to the entire sequence of reactions on the 70S particle leading to the formation of the mature puromycin-reactive P-site complex. And, let us emphasize, binding of thermorubin does not increase the activation barriers noticeably.

The addition of IF creates a completely different situation. In the new 70S·IF$_x$ state, the thermorubin binding and ini-

tiation complex formation sites interact strongly: the binding of thermorubin precludes stable binding of AcPhe-tRNA. The interaction between sites is also strong enough to inhibit binding of fMet-tRNA to 70S particles, directed by poly(A,U,G), when aided by competition from uncharged tRNA. With pure fMet-tRNA, the interaction is only strong enough to produce a decreased steady-state level of fMet-tRNA binding, under conditions where the entire 70S population can be expected to contain bound thermorubin, and where effective dissociation into subunits is at least doubtful, if not excluded.

Poly(A,U,G), unlike poly(U), does not support the formation of AcPhe-tRNA (or fMet-tRNA) 70S initiation complexes unless IF is present. Whatever else the IFs do (e.g., favor P-site binding of fMet-tRNA over AcPhe-tRNA or, in vivo, all the aminoacyl-tRNAs) IF binding must also produce at least one new 70S particle configuration through which the system must pass if binding of poly(A,U,G) is to occur. one would not expect such a transient configuration to bind initiator tRNAs as strongly as either the 70S, poly(U), or poly(A,U,G) end configurations. We propose, as a hypothesis to be tested, that this configuration is the 70S·IF_x·[poly(A,U,G) or poly(U)] constellation in which thermorubin is inhibitory. Certainly, once poly(A,U,G) is well settled into a 70S or even a 30S complex, thermorubin has no further effect, no matter how much twisting or shuttling the other components of the initiation system undergo to arrive at and maintain the final steady state of fMet-tRNA binding. Primarily, the hypothesis reconciles this fact with the pure fMet-tRNA plateau. In any case, thermorubin does not trap the constellation associated with the (IF-3-mediated?) disengagement of UUU or AUG codons from the secondary structure which poly(A,U,G), like natural mRNA, possesses—that process proceeds smoothly, on 30S particles, without interference from thermorubin. Should thermorubin inhibition result from interference with the interaction between poly(A,U,G) and some rRNA sequence [e.g., the U,C-rich sequence near the 3′ end of 16S rRNA (Shine & Dalgarno, 1974)], it would have to be at the point of disengagement of that sequence from its interactions within the 70S particle, since thermorubin, again, inhibits none of the subsequent reactions.

Supplementary Material Available

Appendix I, calculation of the effect of thermorubin on the rate of dissociation of 70S species into subunits and estimation of inhibition of fMet-tRNA binding in the pro-dissociation model, Appendix II, integrated rate expressions for thermorubin inhibition of fMet-tRNA binding in the anti-association model, and Tables V–VII, effects on poly(Phe) synthesis and poly(U) binding (9 pages). Ordering information is given on any current masthead page.

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Glycoproteins in Culture Medium: A Comparison from Cystic Fibrosis and Control Skin Fibroblasts†

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ABSTRACT: High molecular weight glycoproteins have been partially purified by four methods from the medium after growth of human skin fibroblasts. One of these methods, precipitation by heparin, yields one major glycoprotein, M_r 220000, when examined by polyacrylamide gel electrophoresis under denaturing conditions. The glycoprotein was labeled with L-[^{3}H]fucose or D-[^{3}H]glucosamine and yielded glycopeptides after digestion with Pronase. Using this method of precipitation, we made a comparison of matched cystic fibrosis (CF) and control human skin fibroblast media with and without 10% fetal calf serum, a nutritive requirement for growth. The profiles of the [^{3}H]fucose-labeled material from media of both cell types were similar after polyacrylamide gel electrophoresis regardless of the procedure used to obtain them. In contrast, differences were seen in the composition of the material obtained by precipitation with heparin, which suggested differences in glycosylation by CF fibroblasts. In addition, differences were noted which were related to the presence or absence of fetal calf serum in the growth medium. The analysis showed that when the CF and control materials were compared, the heparin precipitate of the CF medium without fetal calf serum contained (1) less protein and carbohydrate, (2) 30–35% less radioactivity per milligram of protein, (3) a lowered fucose content, (4) higher sialic acid content in confluency, and (5) lower turnover when expressed as counts per minute per nanomole of fucose. When the medium from the CF fibroblasts contained fetal calf serum, the heparin-precipitated material had more radioactivity and a higher ratio of fucose to other monosaccharides than that from control cells. Thus, there was either a differential response of the CF and control fibroblasts grown in the presence of 10% fetal calf serum or a differential recruitment of other glycoproteins during the precipitation of the serum-containing medium. The results are discussed in relation to previous findings of the monosaccharide content of CF fibroblast membranes and CF secretions.

Glycoproteins of high molecular weight are found associated with the cell surface or in the growth medium of fibroblasts and other cell types (Glick & Flowers, 1978). The relationships of these glycoproteins, for example, fibronectin, to biological functions such as cell adhesion, morphology, transformation, and embryogenesis have been reviewed (Vaheri & Mosher, 1978; Frazier & Glaser, 1979; Kobata, 1979; Yamada et al., 1980). The complexity of the interactions of high molecular weight glycoproteins with glycosaminoglycans and other macromolecules has been reported (Ruoslahti et al., 1980). Another glycoprotein, Excitoporin, was reported to be associated with neurites and membranes of differentiating mouse (Littauer et al., 1980) or human (Littauer et al., 1979) neuroblastoma cells in culture. When the cells were not differentiated, a glycoprotein of similar size subunits was found in the culture medium. Whether or not the glycoproteins in the culture medium represent material shed from the membrane and/or internal products secreted from the cells is not fully understood. The difficulty in purifying these glycoproteins for characterization has impaired progress in this area (Glick & Flowers, 1978; Kobata, 1979; Momoi et al., 1980).

Membrane glycoproteins from skin fibroblasts of patients with cystic fibrosis (CF)[1] have an altered carbohydrate composition when compared to those of age-, race-, and sex-matched controls (Scanlin & Glick, 1977). That is, the molar ratios of Fuc:NeuAc:Gal:Man were 1:3:4:2 and 1:4:7:5 in the membrane glycopeptides of CF and control fibroblasts, respectively. Since this group of glycopeptides is externally oriented toward the environment of the cells (Glick, 1979), it was of interest to isolate the glycoproteins from the growth medium and determine if an alteration exists also in these glycoproteins.

We report here methods for the isolation and a partial characterization of high molecular weight glycoproteins from the culture medium of human skin fibroblasts. A comparison of these glycoproteins from the medium after growth of CF and matched control fibroblasts is presented.

Materials and Methods

Materials. Sodium heparin was purchased from SchwarzMann; CNBr-activated Sepharose and heparin–Sepharose were from Pharmacia. L-[5,6-$^3H_2$]Fucose (60 Ci/mmol), L-[1,5,6-$^3H_3$]fucose (3 Ci/mmol), D-[6-^{3}H]glucosamine (19 Ci/mmol), L-[U-^{14}C]proline (294 mCi/mmol), and

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[1] Abbreviations: CF, cystic fibrosis; FCS, 10% fetal calf serum; NaDodSO$_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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