CHAPTER 3

RESULTS

Determination of JW101 and JW102 plasmids.

Both α JW101 and β JW102 are recombinant DNAs which have the same size (6.1 kb) and resist to the same antibiotic (tetracycline). After purification these two plasmids could be characterized by digestion with Bam H1 endonuclease enzyme. Plasmid JW101 was almost totally converted to linear DNA (6.1 kb) because of the presence of a single Bam H1 site (figure 2 lane C). In contrast the plasmid JW102 which has two Bam H1 sites showed two distinct DNA fragments, after digestion with Bam H1 enzyme (lane E). (Wilson et al, 1978).

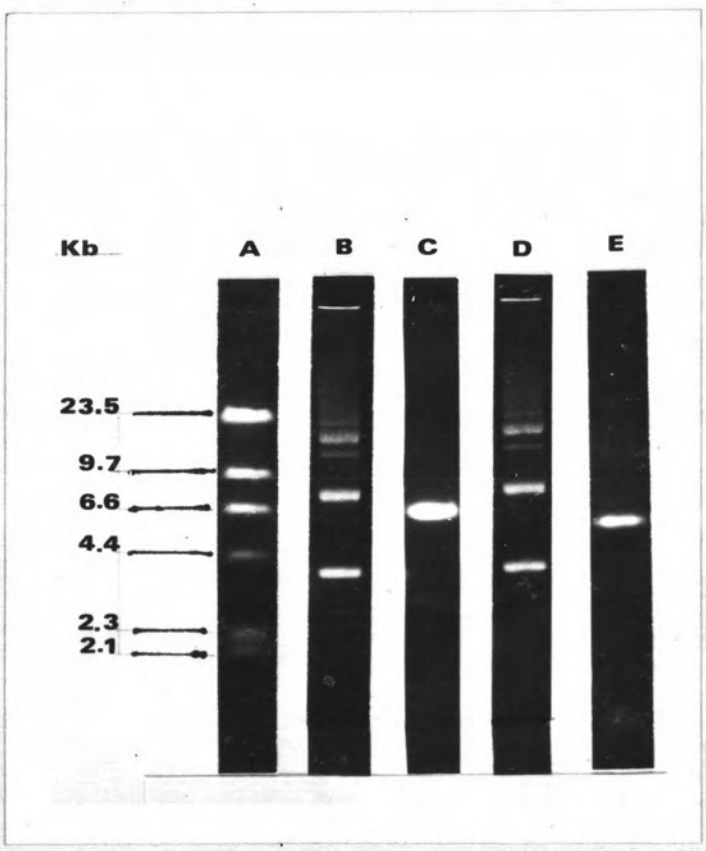


Figure. 2 Determination of α JW101 and β JW102 plasmid by Bam Hl restriction enzyme.

A λ Hind III marker

B JW101 plasmid

C JW101 + Bam Hl

D JW102 plasmid

E JW102 + Bam Hl

Purification of plasmid DNA

α JW101 and β JW102 extracted by the rapid alkali extraction method contained many forms of plasmid (superhelical relax and multimers forms) ribosomal RNA and contamination of chromosamal DNAs. Agarose gel electrophoresis was used to separate these different DNAs and RNAs as shown in Figure 3 (lane B and lane D). Superhelical and Relax forms of these two plasmid were obtained after trapping with DEAE paper (Figure 3 lane C and lane E).

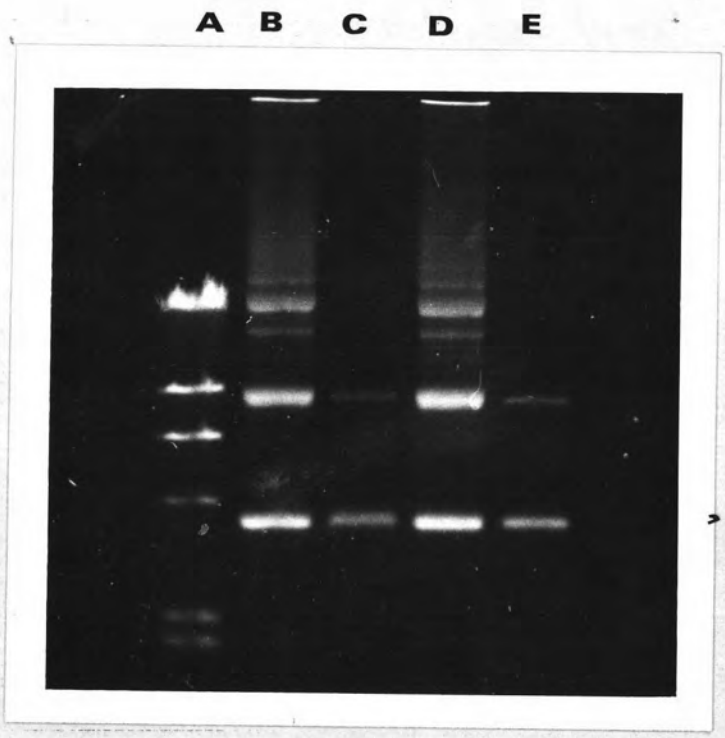


Figure 3. Different forms of plasmid DNA separated by 0.7% agarose gel electrophoresis.

A λ Hind III

B JW101 plasmid

C Purified JW101

D JW102 plasmid

E Purified JW102

Labelling DNA by nick translation

Labelling α JW101 and β JW102 were used to detect the α and β globin mRNA respectively. Kinetics followed by TCA precipitation on Whatman GF/A filters was shown in Figure 4. Rate of nucleotide incorparation was high at about 90 min after initiating the reaction, then dropped. The incorporated plasmid was separated by Sephadex G-50 and eluted by TE buffer before the unincorporated nucleotides (Figure 5). The efficiency of this nick translation system was usually about 40%.

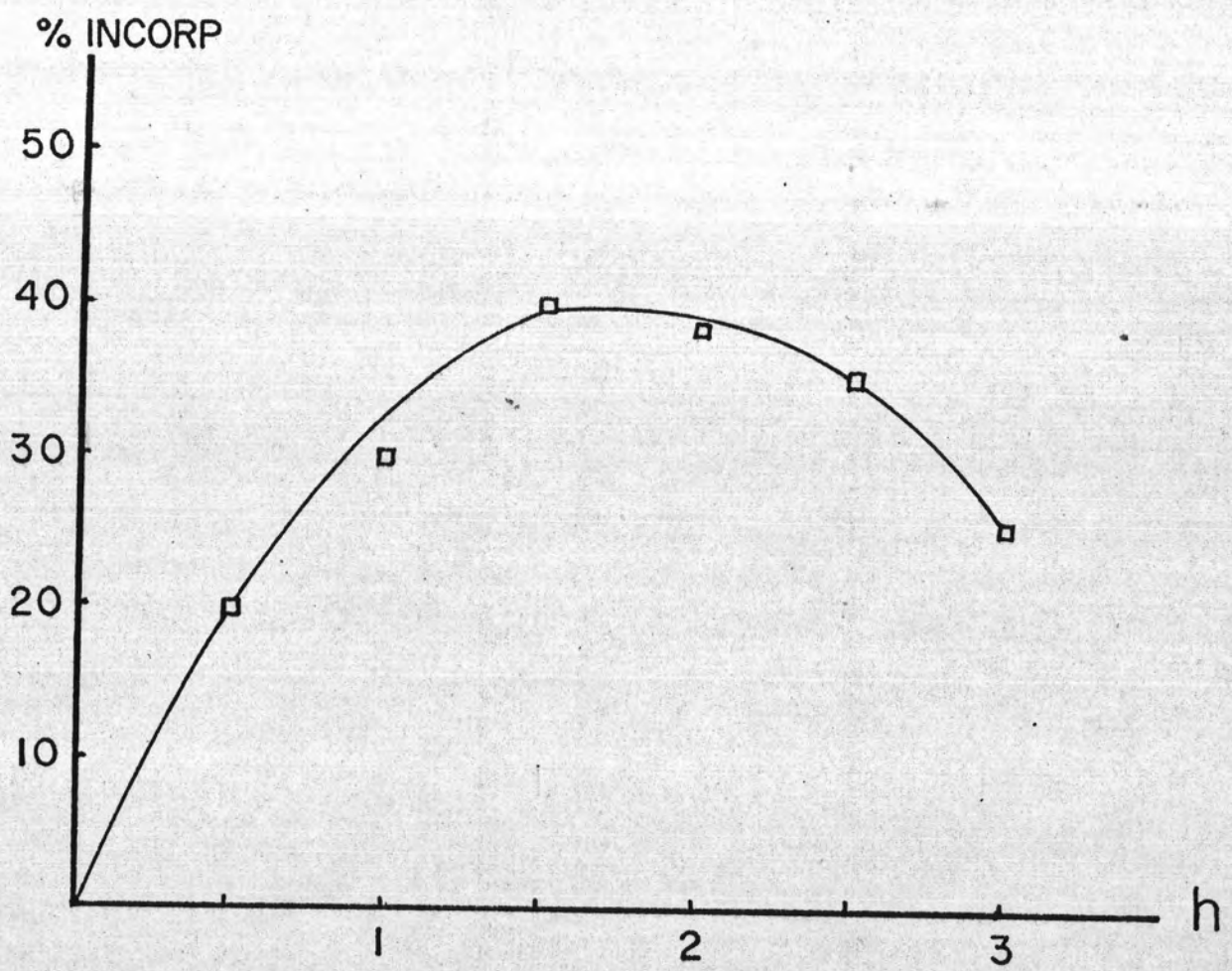


Figure 4 Time course of nick translation reaction.

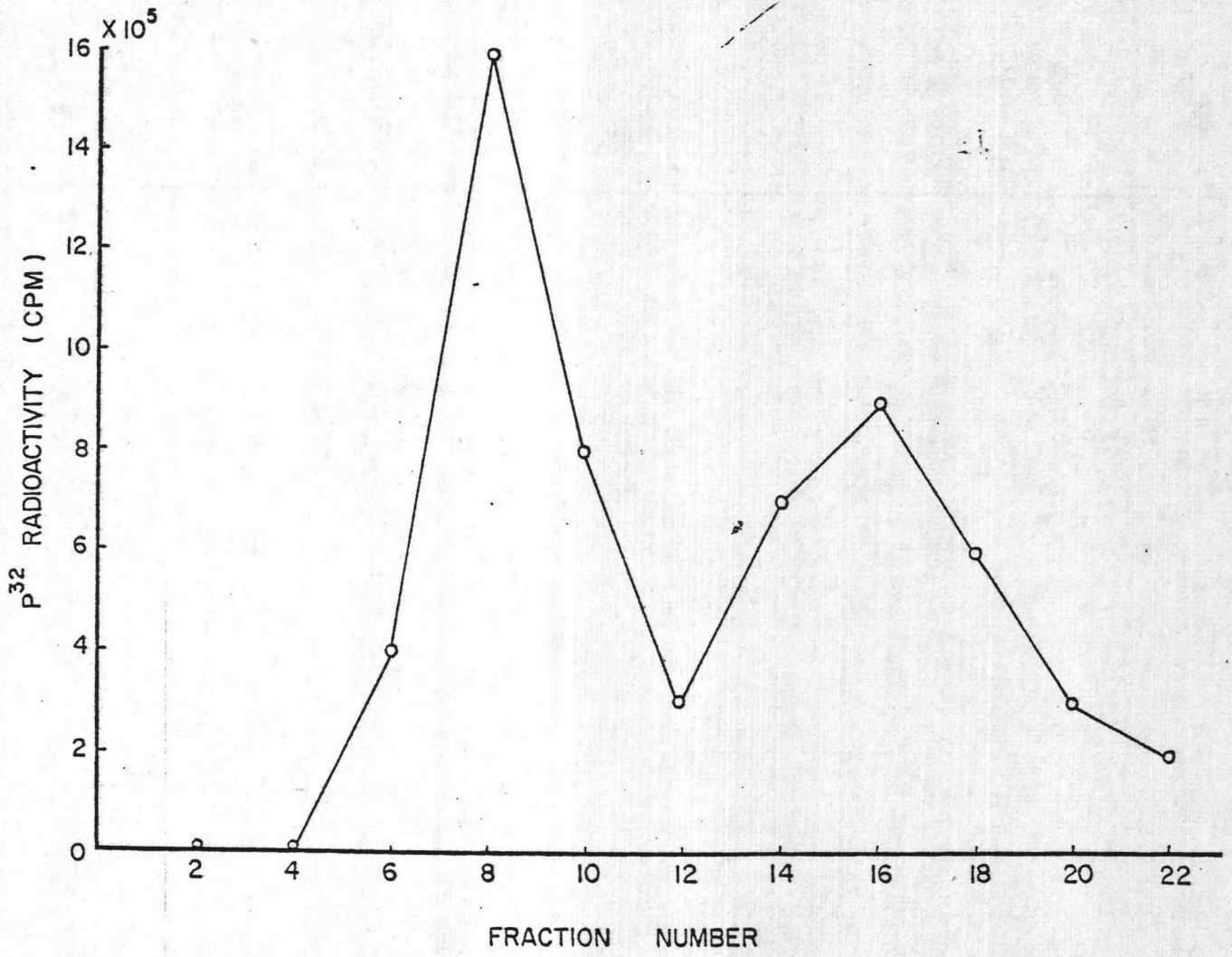


Figure 5 Seperation of labeled plasmid DNAs and the free nucleotides by Sephadex G-50 column chromatography.

Analysis of total cellular RNA

A. NaOH hydrolysis

Cellular RNA treated with 0.1 M NaOH showed complete hydrolysis (lane B, Figure 6.) whereas DNA was not hydrolysed by NaOH (lane D, Figure 6.)

B. Spectrophotometric Determination of RNA

Spectrophotometric measurement of the amount of UV irradiation absorbed by the base such as RNA is simple and accurate. The amount of the RNA was measured at wavelength 260 nm and 1 OD_{260} is 35 μg/ml RNA. In addition purification of the RNA was determined by the ratio between the readings at 260 nm and 280 nm (OD_{260}/OD_{280}).

Figure 7. (A) shows the pure RNA which OD_{260}/OD_{280} was 2.0.

If there is contamination with protein or phenol the OD_{260}/OD_{280} will be significantly less than 2.0 (Figure 7. B and C).

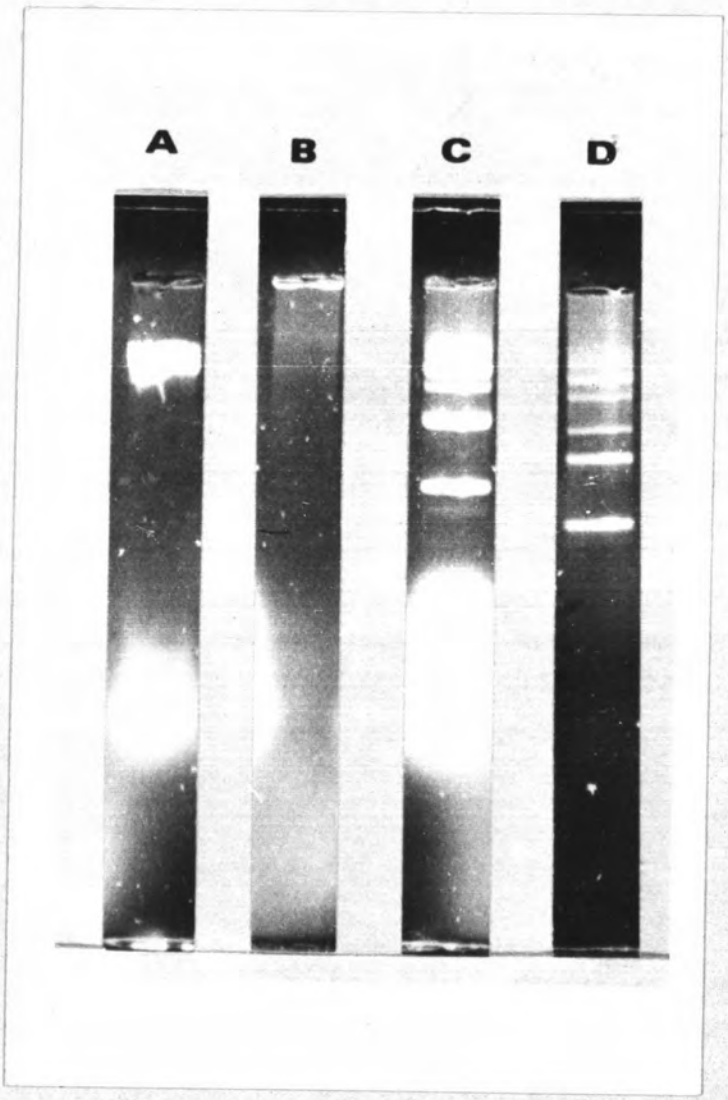


Figure 6. Analysis of the total cellular RNA by NaOH hydrolysis.

A Total RNA

B RNA + NaOH

C JW101 plasmid

D JW101 + NaOH

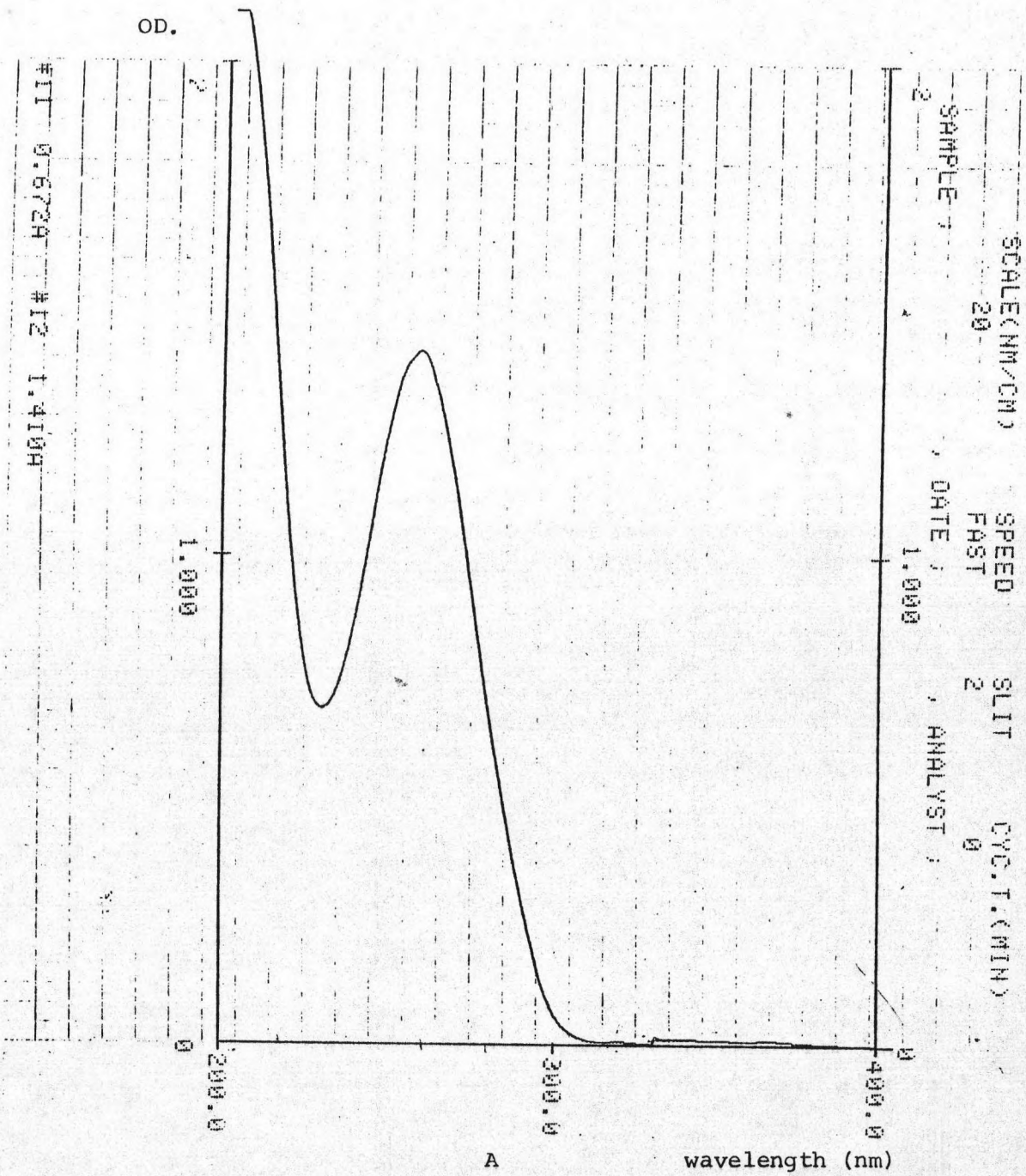


Figure 7. Spectrophotometric Determination of total RNA

A. pure RNA

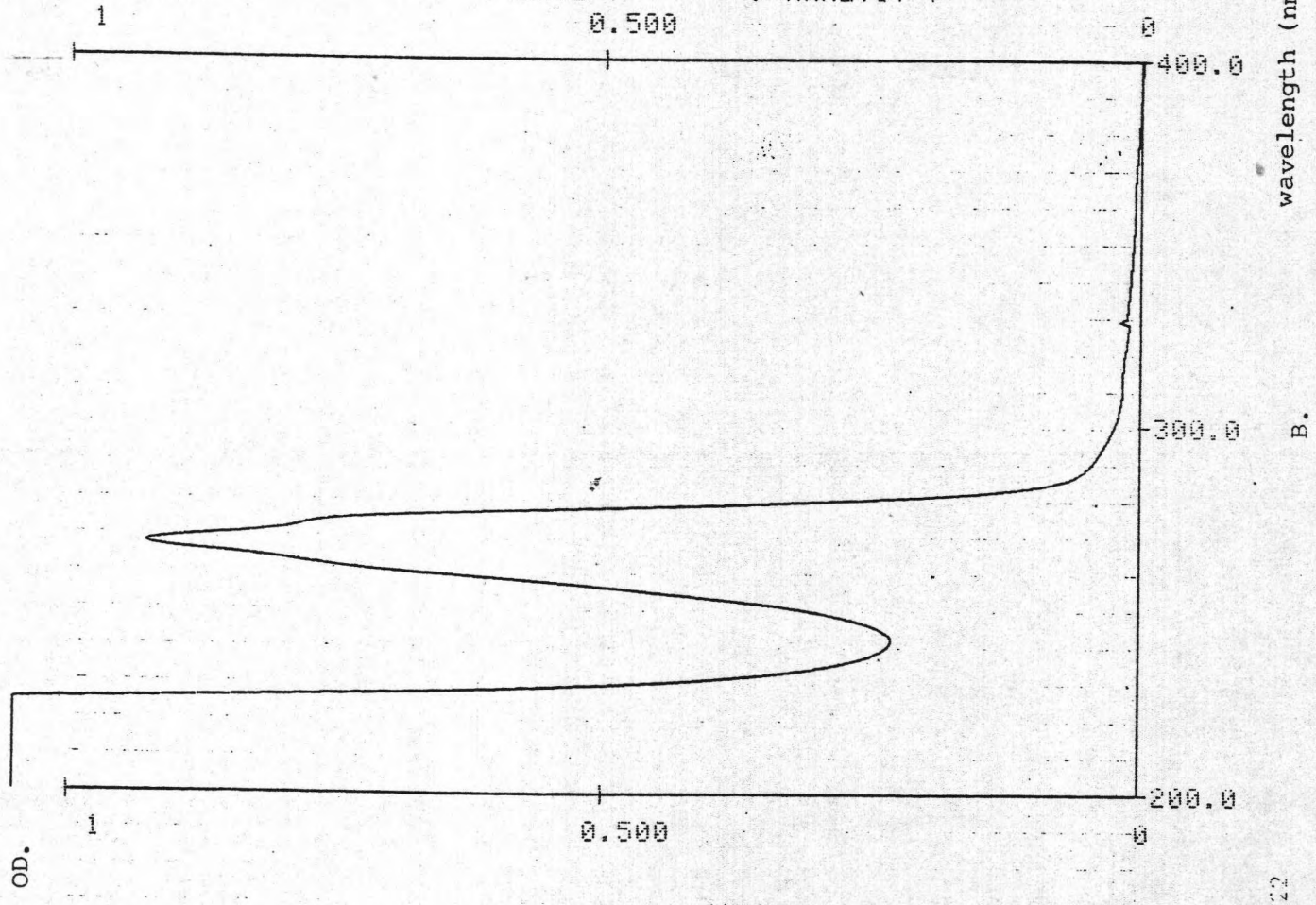
B. Contamination with phenol

C. Contamination with protein.

RANGE	LOW	HIGH	START WL(NM)	END WL(NM)
ABS.	0	1	400	200

SCALE(NM/CM)	SPEED	SLIT	CYC.T.(MIN)
20	FAST	2	0

SAMPLE ; , DATE ; , ANALYST ;



#23 0.383A #24 0.605A

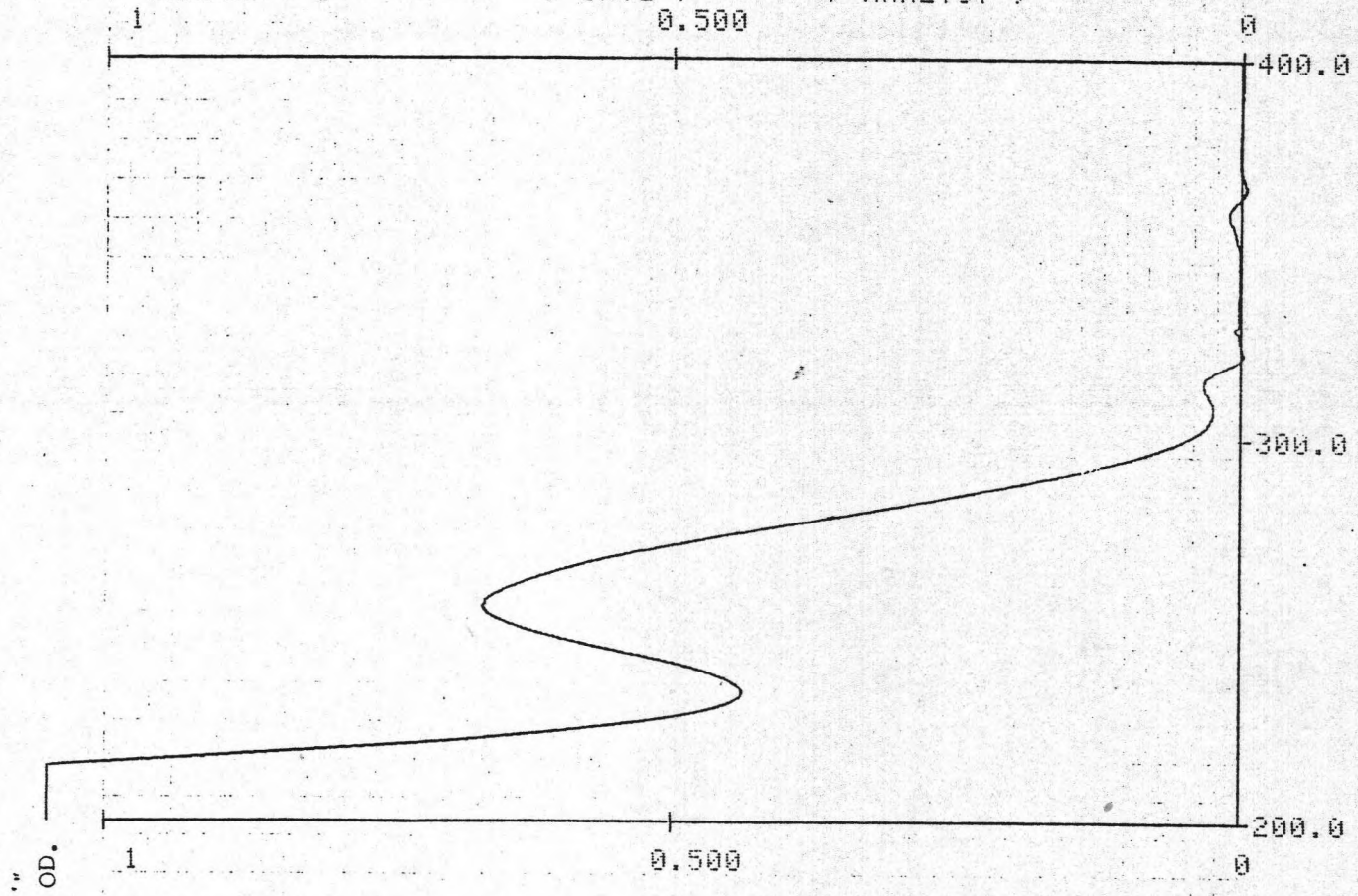
KF 200-91522

#05 0.652A #06 0.379A

RANGE	LOW	HIGH	START WL(NM)	END WL(NM)
ABS.	0	1	400	200

SCALE(NM/CM)	SPEED	SLIT	CYC.T.(MIN)
20	FAST	2	0

SAMPLE ; , DATE ; , ANALYST ;



wavelength (nm).

Comparison of the hot phenol and cold phenol extraction of RNA.

RNA was extracted by the hot phenol and cold phenol method from the same amount of packed red cell (4 ml). The amount of the RNA obtained by hot phenol extractions was about 19-26% of the total RNA measured by pentose analysis. The yield of RNA was decreased to about 6.5-15% of the total RNA when the cold phenol extraction was used (Table 1). Thus the amount of RNA extracted by hot phenol method was about 2-3 times higher than those extracted by cold phenol method.

Table 1. Comparison of the yields of total RNA extracted by hot and cold phenol method.

		Total RNA from Pentose analysis	Hot phenol method		Cold phenol	
Sample	No	µg/ 4·ml cells	µg/4ml cells	%	µg/4ml cells	%
	1	624	152	24	60	9.6
normal	2	640	180	28	42	6.5
	3	661	190	28	100	15
		641 ± 18.5	174 ± 19.6	26±2.3	67±29	10±4.3
	1	1088	296	27	140	9.9
α-thal$_1$/	2	2026	433	21	302	14
α-thal$_2$	3	1066	264	24	101	9.4
		1393±548	331±89	24±3	181±10.6	11.1±2.5
	1	1717	326	19	173	10
α-thal$_1$/	2	2060	424	20	161	7.8
Hb CS	3	1663	324	19	241	14.4
		1813±21.5	358±57	19±0.5	191±43	8.3±5.5

Binding and retention of RNA on nitrocellulose paper.

The glyoxalated RNA was retained on nitrocellulose about 42% during the process of prehybridization, hybridization, non stringent wash and stringent wash. The results were the same among the samples of normal, α-thal$_1$/ α-thal$_2$ and α-thal$_1$/Hb CS. Different amounts of RNA applied to the nitrocellulose also gave the same retention of RNA on the paper (Table 2).

The loss of RNA on nitrocellulose occured mostly during the process of prehybridization about 43% of total RNA. Small amounts of RNA were loss during the process of hybridization, non stringent wash and stringent wash (13%, 1% and 0.13%, respectively).

Table 2. RNA retention on nitrocellulose after processing of hybridization.

Sample	No	RNA content µg.	% retention	average.
normal	I	1	42.3	42.07 ± 0.43
		3	42.5	
		6	42.0	
		10	41.5	
	II	1	42.0	42.22 ± 0.33
		3	42.7	
		6	42.2	
		10	42.0	
	III	1	42.0	42.00 ± 0.24
		3	41.7	
		6	42.0	
		10	42.3	
α-thal$_1$/ α-thal$_2$	I	1	42.0	42.22 ± 0.35
		3	42.3	
		6	42.7	
		10	41.9	
	II	1	43.0	42.6 ± 0.32
		3	42.2	
		6	42.6	
		10	42.6	
	III	1	42.0	42.37 ± 0.43
		3	42.3	
		6	42.2	
		10	43.0	
α-thal$_1$/ Hb CS	I	1	42.3	42.65 ± 0.43
		3	42.3	
		6	43.2	
		10	42.8	
	II	1	43.0	42.77 ± 0.26
		3	42.5	
		6	42.6	
		10	43.0	
	III	1	42.1	42.25 ± 0.33
		3	43.2	
		6	42.3	
		10	41.4	

Sensitivity of hybridization of RNA on nitrocellulose paper.

After baking, the gloxalated RNA blot was treated with 200 ml 20 mM Tris pH 8.0 at 100°c for 10 minute to increase the sensitivity of DNA-RNA hybridization. This treatment will remove glyoxal from the denatured RNA.

Figure 8 showed that the hybridization of α JW101 to the denatured RNA without glyoxal was 2 folds higher than the glyoxalated RNA. glyoxalated

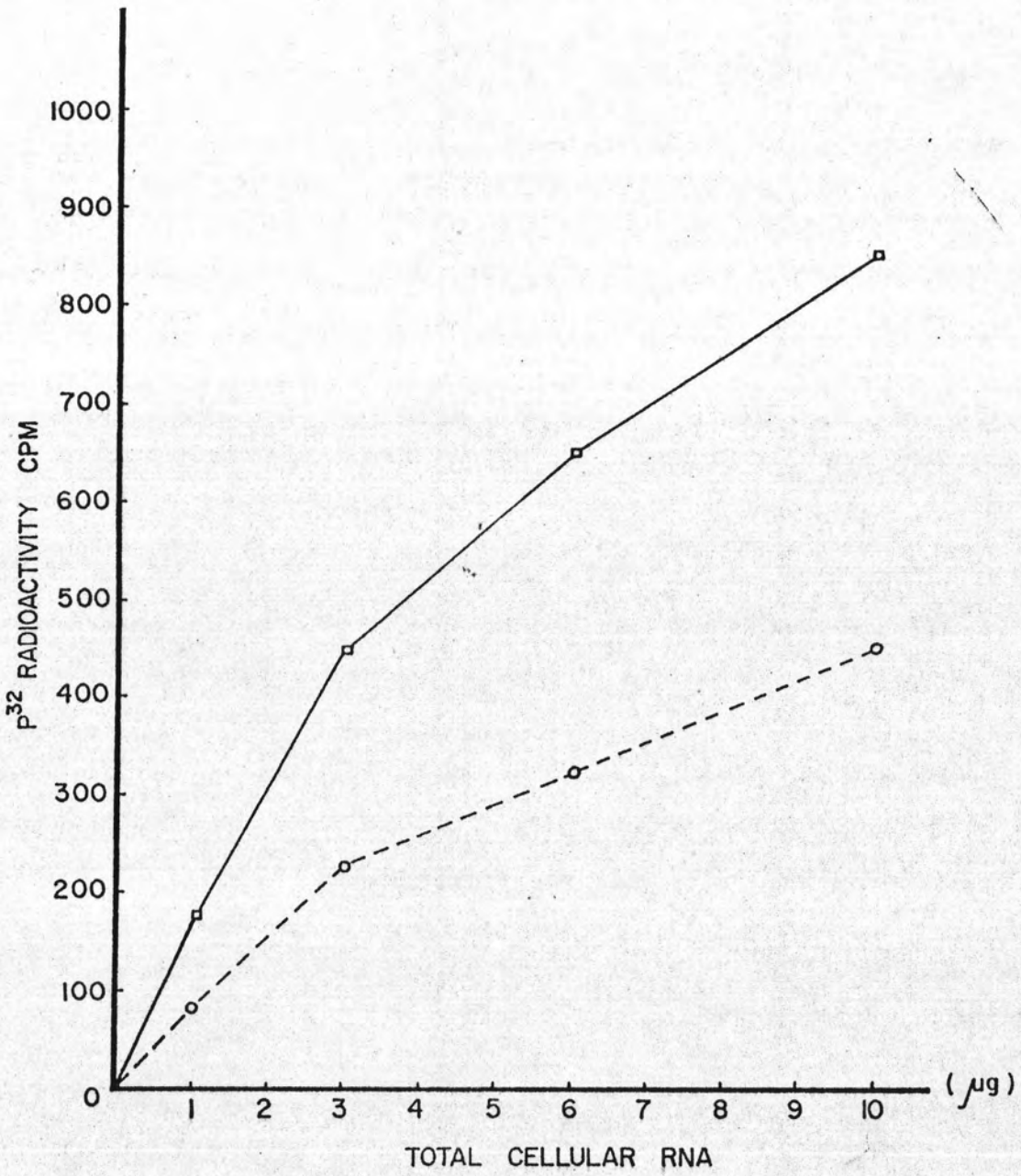


Figure 8. A Comparison of the hybridization of α JW101 to cellular RNA with the glyoxal (0-----0) and without the glyoxal (0———0).

Hybridization of α JW101 to total cellular RNA extracted by hot phenol method and cold phenol method.

RNA extracted by hot phenol and cold phenol method showed the same hybridization to α JW101 probe. Kinetics of the hybridization at different concentrations of RNA were shown on Figure 9.

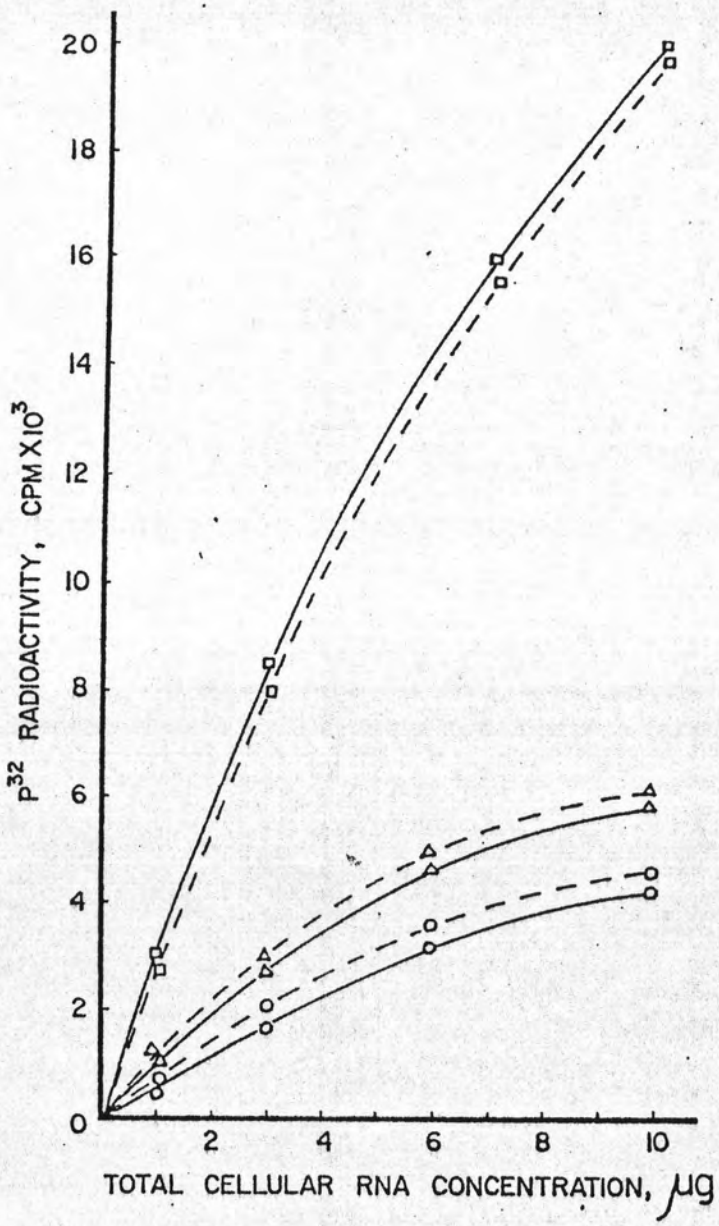


Figure 9 Comparison of hybridization of total RNA from the hot phenol extraction and cold phenol extraction to α JW101 recombinant plasmid.

□ Normal

Δ α-thal$_1$/ α-thal$_2$

○ α-thal$_1$/ Hb CS.

----- RNA from hot phenol extraction

——— RNA from cold phenol extraction

Hybridization of the total RNA to α JW101 or β JW102 plasmid

Hybridization of α JW101 or β JW102 to the total RNA concentration of 1,3,6,10 µg prepared from peripheral blood of a normal individual (□ -----□), a patient with Hb H disease α-thal$_1$/α-thal$_2$ (Δ ----- Δ) and α-thal$_1$/Hb CS (O ----- O) to compare the amount of α or β-globin mRNA respectively. The results show that α mRNA of normal individual was 3-4 fold higher than Hb H disease and Hb H α-thal$_1$/ α-thal$_2$ was higher than α-thal$_1$/Hb CS (Figure 10 A.)

For the level of amount of β mRNA, it was found that normal individual, Hb H α-thal$_1$/α-thal$_2$and α-thal$_1$/Hb CS have the same amount of β mRNA (Figure 10 B.)

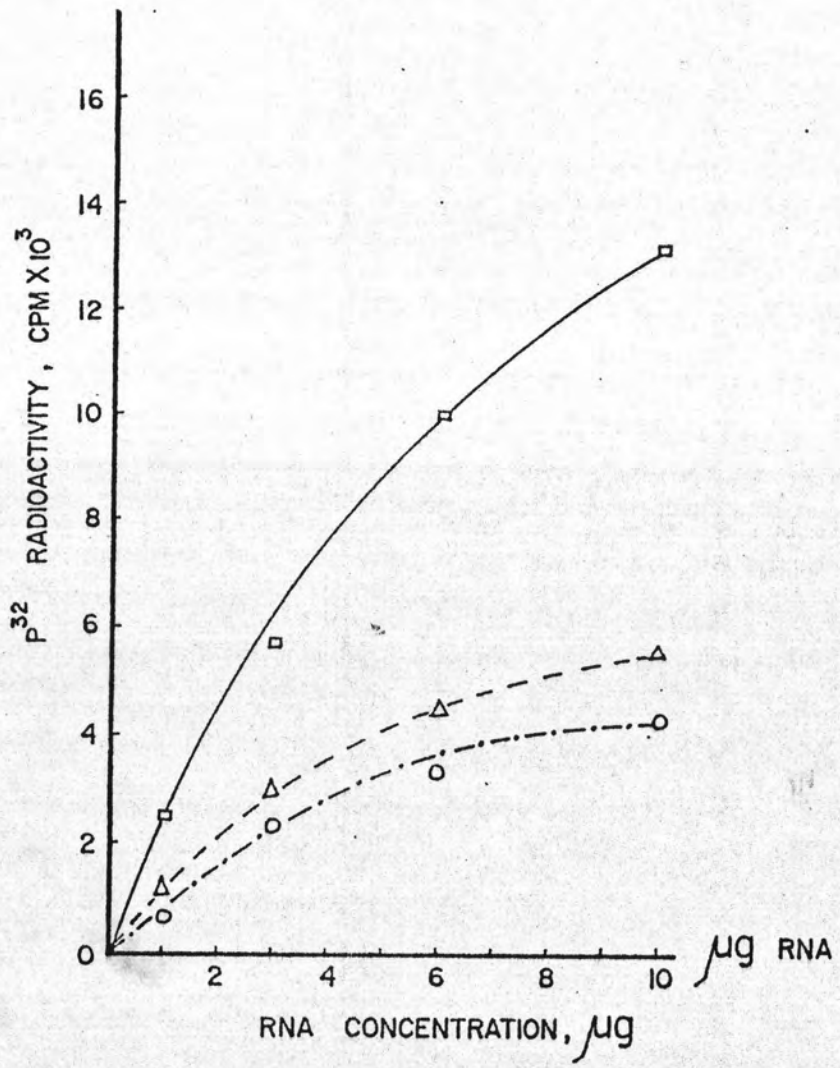


Figure 10A. Hybridization of varying amounts of total RNA from the peripheral blood of a normal individual, individuals with Hb H disease to α JW101 recombinant plasmid.

□——□ Normal

Δ ----- Δ α-thal$_1$/ α-thal$_2$

O - - - O α-thal$_1$/ Hb CS.

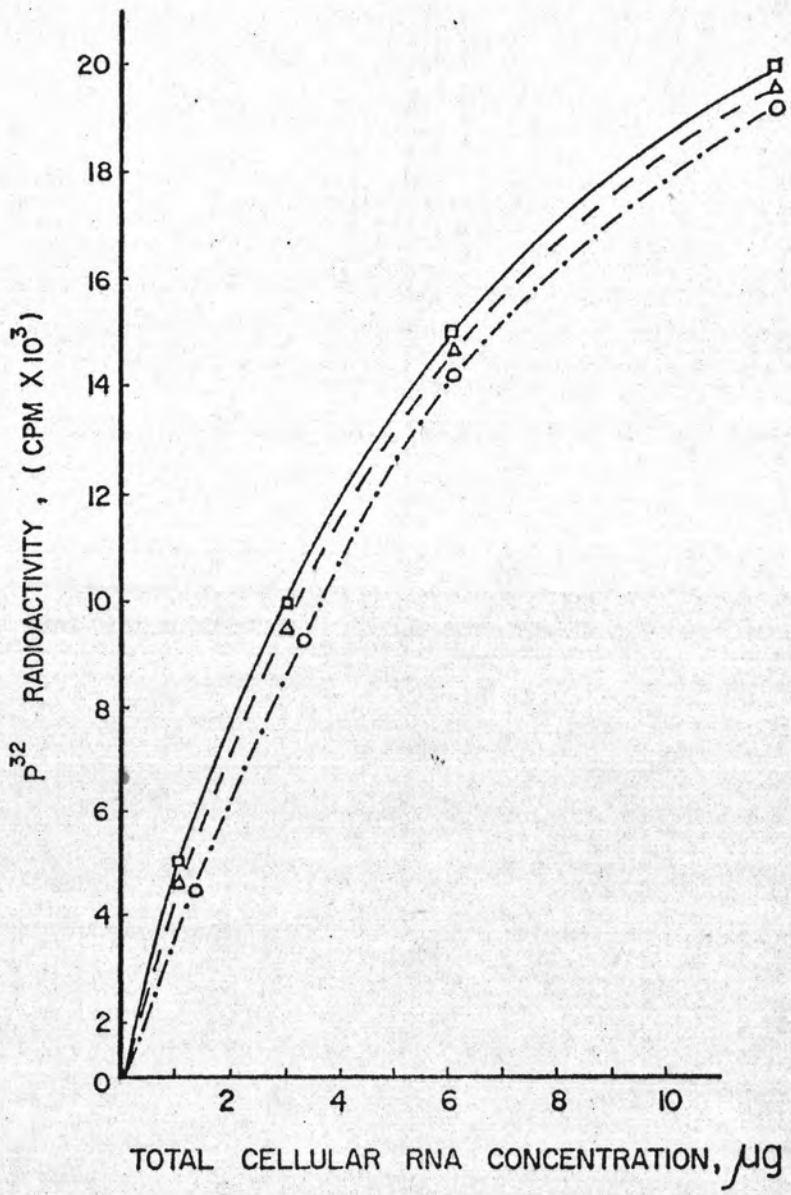


Figure 10B Hybridization of varying amounts of total RNA from the peripheral blood of a normal individual, individuals with Hb H disease to β JW102 recombinant plasmid.

□——□ normal

Δ ----- Δ α-thal$_1$/ α-thal$_2$

O -·-·- O α-thal$_1$/ Hb CS.

Comparison amount of α and β globin mRNA between the two types of Hb H disease.

To measure the amount of α globin mRNA, the labelled radioactive JW101 was hybridization to various concentrations of total cellular RNA and the level of α globin mRNA was determined by CPM of radioactivity from the hybridization. Table 3 and Figure 11 show the comparison amount of α globin mRNA between Hb H disease $\alpha\text{-thal}_1/\alpha\text{-thal}_2$ (12 samples), $\alpha\text{-thal}_1$/Hb CS (9 samples) and normal individuals (4 samples) at 1,3,6,10 total cellular RNA concentration. The data show the means level of α globin mRNA in normal individuals are higher than Hb H diseases and Hb H diseases $\alpha\text{-thal}_1/\alpha\text{-thal}_2$ are higher than $\alpha\text{-thal}_1$/Hb CS in every concentration of total RNA.

For the level of β globin mRNA in these samples (Table 4 and Figure 12) are the same level.

The α/β ratios was used to determined the level of α globin mRNA and it was calculated from the ratio of

$$\frac{\text{CPM of } \alpha \text{ JW101 hybridization to total RNA}}{\text{CPM of } \alpha \text{ JW102 hybridization to total RNA}}$$

In the table 5 and Figure 13 the means of α/β mRNA ratio of the $\alpha\text{-thal}_1/\alpha\text{-thal}_2$ are higher than $\alpha\text{-thal}_1$/Hb CS. It is indicating that the RNA in the $\alpha\text{-thal}_1/\alpha\text{-thal}_2$ samples contains more α specific sequences relative

to β sequences, when compare to RNA from α-thal$_1$/Hb CS. The average value of the α/β mRNA ratio of α-thal$_1$/ α-thal$_2$ and α-thal$_1$/Hb CS is 0.426±0.04 and 0.335±0.07 respectively. However, there is some α/β values overlap between these two types of Hb disease. By applying student's t test for small samples the differences in ratios observed between these two types of Hb H diseases are significant at 99.99% confident level.

Table 3. The level of α mRNA measured by CPM from the hybridization of labelled radioactive α JW101 to difference concentration of total cellular RNA in normal individuals and individuals with Hb H disease.

Sample	No.	CPM in differences concentration of total RNA. 1 μg	3 μg	6 μg	10 μg
	1	1264	2570	4347	5291
	2	783	1381	1697	3521
	3	1519	3663	6060	6666
α-thal$_1$/	4	1633	4405	7194	7874
Hb CS	5	1579	3521	6535	6944
	6	858	2309	3401	4273
	7	1308	3436	4629	5128
	8	1193	3030	6802	7739
	9	1095	2500	3676	4587
		1248 ± 302	2979 ± 894	4926 ± 1846	5780 ± 1574
	1	1321	2624	4761	6756
	2	1414	3076	5586	7246
	3	1666	3460	6666	7874
	4	1512	3134	5780	9009
α-thal$_1$/	5	1712	3816	7042	8547
α-thal$_2$	6	1689	3412	6802	9009
	7	1686	4405	7575	8929
	8	1958	3993	6993	10309
	9	1923	4716	6535	6578
	10	1845	3968	7352	9259
	11	1138	2770	4975	7142
	12	1161	2824	3968	6849
		1585 ± 277	3516 ± 669	6169 ± 1141	8125 ± 1211
	1	5494	16129	28571	32258
normal	2	7812	18860	31250	37030
	3	4255	11111	15384	18658
	4	3772	8474	22727	22777
		5333 ± 1804	13643 ± 4709	24483 ± 7032	27680 ± 8442

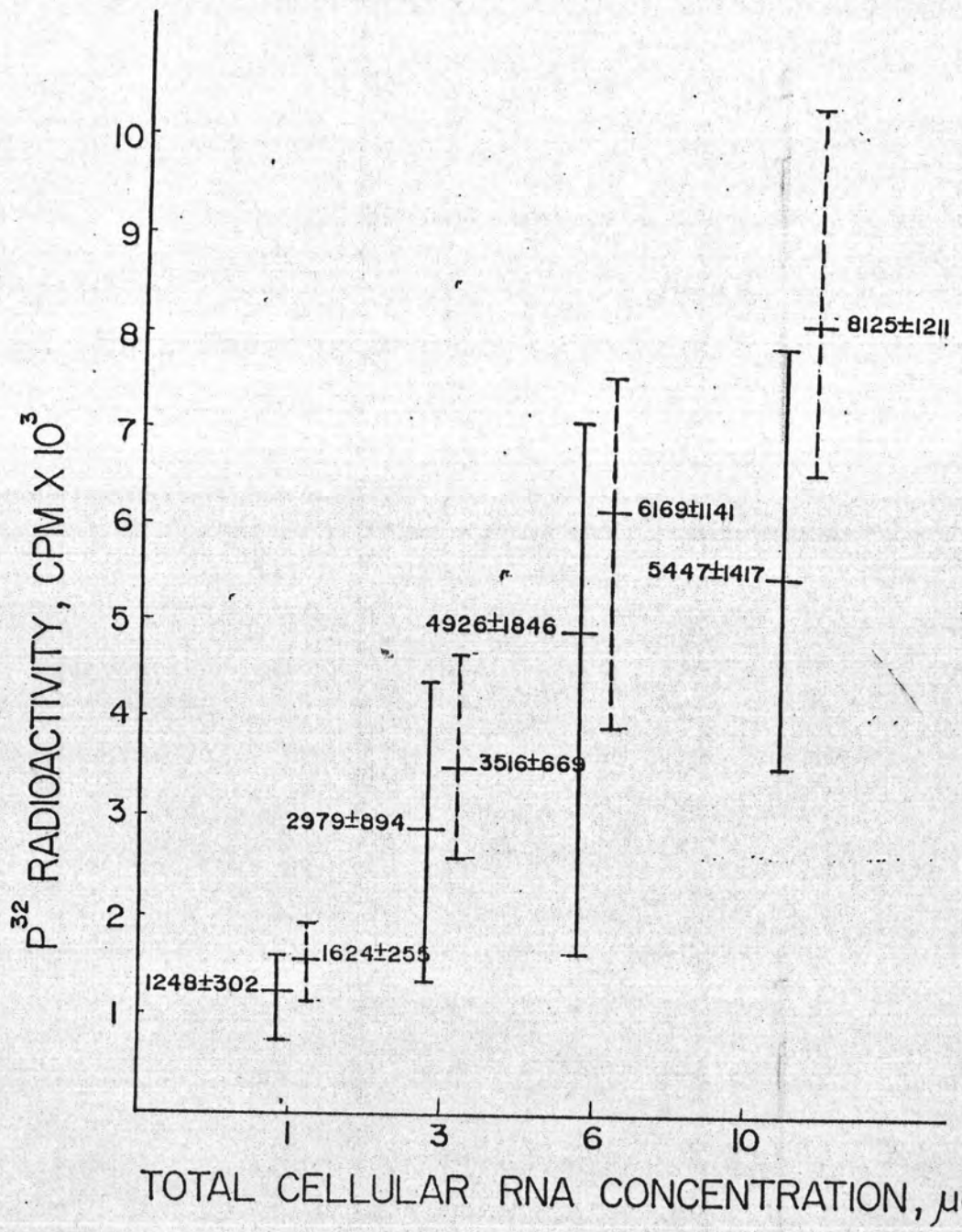


Figure 11 The mean of α mRNA level in total cellular RNA at various concentration.

——— α-thal$_1$/Hb CS

----- α-thal$_1$/ α-thal$_2$

Table 4. The level of β mRNA measured by CPM from the hybridization of labelled radioactive β JW102 to differences concentration of total cellular RNA in nomal individuals and individual with Hb H disease.

Sample	No.	CPM in differences concentration of total RNA.			
		1 µg	3 µg	6 µg	10 µg.
	1	3579	7330	12450	15117
	2	3450	6077	8713	16130
	3	3632	9157	15538	15999
α-thal$_1$/	4	3789	8931	17540	20189
Hb CS	5	3881	9265	16300	18767
	6	3555	8246	16195	17434
	7	3675	9544	13510	16545
	8	3562	8965	17432	22111
	9	3364	7575	12253	15010
		3609 ± 159	8343 ± 1142	14436 ± 2930	17478 ± 2424
	1	3336	6299	12335	17111
	2	3404	7720	14132	17102
	3	3521	7966	13987	18117
	4	3544	8035	14507	20512
	5	3666	8990	15644	19893
α-thal$_1$/	6	3720	7600	15101	20015
	7	3519	8792	15781	19842
α-thal$_2$	8	3924	8680	15202	18945
	9	3768	9624	13775	15250
	10	3791	8817	15999	19697
	11	3121	7486	15320	18794
	12	3256	7844	11670	18764
		3547 ± 238	8154 ± 879	14454 ± 1359	18670 ± 1540
normal	1	2859	7893	14285	16129
	2	3444	8897	16525	19515
	3	3010	7673	14120	15230
	4	2950	7013	11340	13340
		3065 ± 259	7869 ± 780	14067 ± 2123	16053 ± 2583

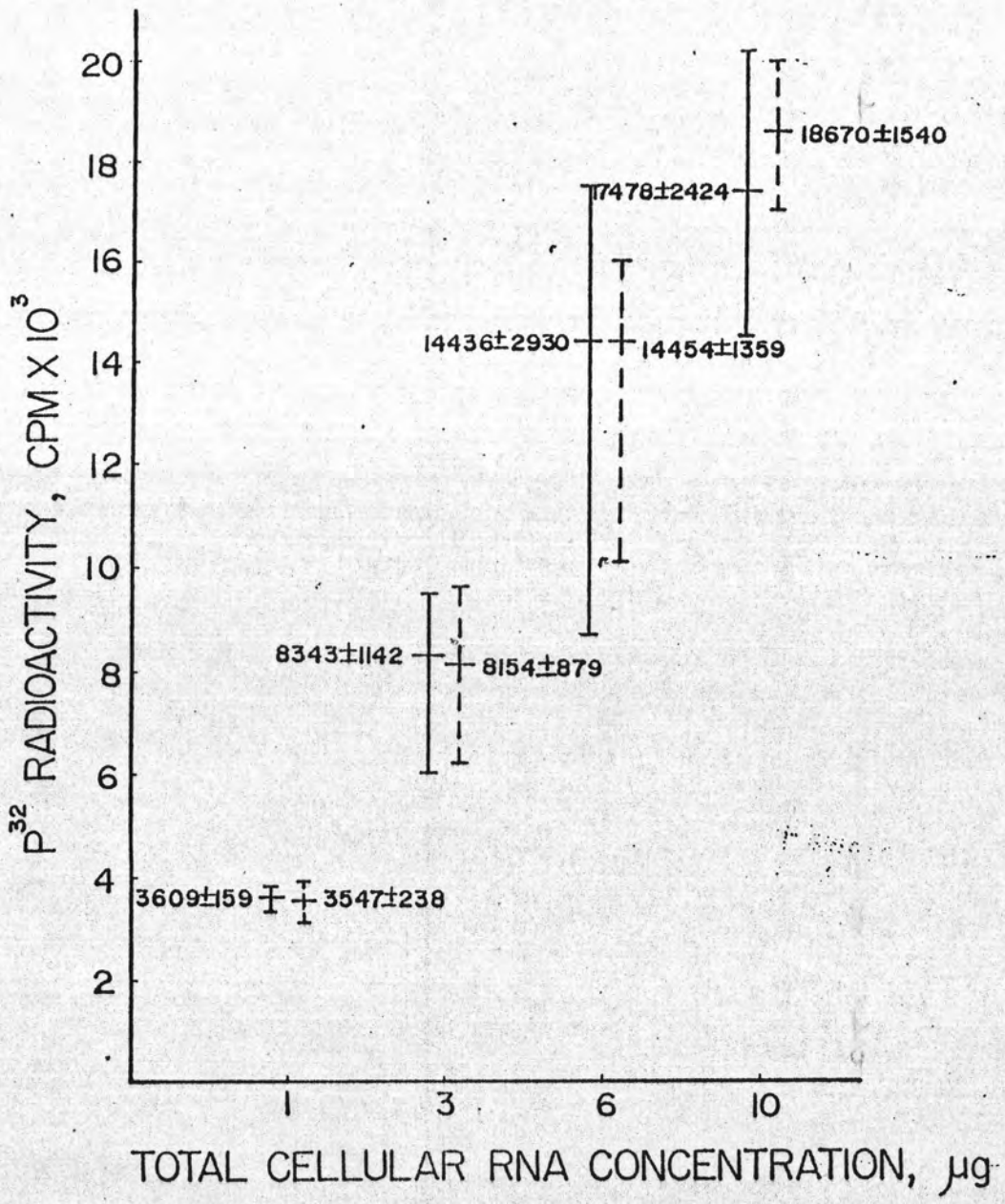


Figure 12 The mean of β mRNA level in total cellular RNA at various concentration.

——— α-thal$_1$/Hb CS

----- α-thal$_1$/ α-thal$_2$

Table 5. α/β globin - specific RNA ratios calculatated by CPM from hybridization of α JW101 and CPM from hybridization of β JW102 in total RNA from peripheral blood of individuals with Hb H disease.

		α/β ratios in differences concentration of total RNA.				
Sample	No.	1 μg	3 μg	6 μg	10 μg	average
	1	0.35	0.35	0.34	0.35	0.347
	2	0.22	0.22	0.19	0.21	0.210
	3	0.41	0.40	0.39	0.41	0.404
	4	0.43	0.49	0.41	0.39	0.430
α-thal$_1$/	5	0.40	0.38	0.40	0.37	0.387
	6	0.24	0.28	0.21	0.24	0.242
Hb CS	7	0.35	0.36	0.34	0.30	0.337
	8	0.33	0.33	0.39	0.35	0.350
	9	0.32	0.33	0.30	0.30	0.312
Mean±SD		0.34 ± 0.07	0.34 ± 0.07	0.33 ± 0.08	0.32 ± 0.06	0.335 ± 0.07
	1	0.39	0.41	0.38	0.39	0.392
	2	0.41	0.39	0.39	0.42	0.402
	3	0.47	0.43	0.47	0.43	0.450
	4	0.42	0.39	0.39	0.43	0.407
	5	0.46	0.42	0.45	0.42	0.437
α-thal$_1$/	6	0.45	0.45	0.45	0.42	0.45
	7	0.47	0.50	0.48	0.45	0.47
α-thal$_2$	8	0.46	0.46	0.46	0.54	0.48
	9	0.51	0.49	0.47	0.43	0.47
	10	0.48	0.45	0.45	0.47	0.46
	11	0.36	0.37	0.32	0.38	0.35
	12	0.35	0.36	0.34	0.36	0.35
Mean±SD		0.43 ± 0.05	0.42 ± 0.04	0.42 ± 0.05	0.43 ± 0.04	0.426 ± 0.04
	1	1.92	2.04	2.00	2.00	1.99
	2	2.26	2.11	1.89	1.89	2.03
Normal	3	1.41	1.44	1.08	1.22	1.30
	4	1.27	1.20	2.00	1.70	1.54
Mean±SD		1.71 ± 0.45	1.69 ± 0.44	1.74 ± 0.44	1.70 ± 0.34	1.71 ± 0.35

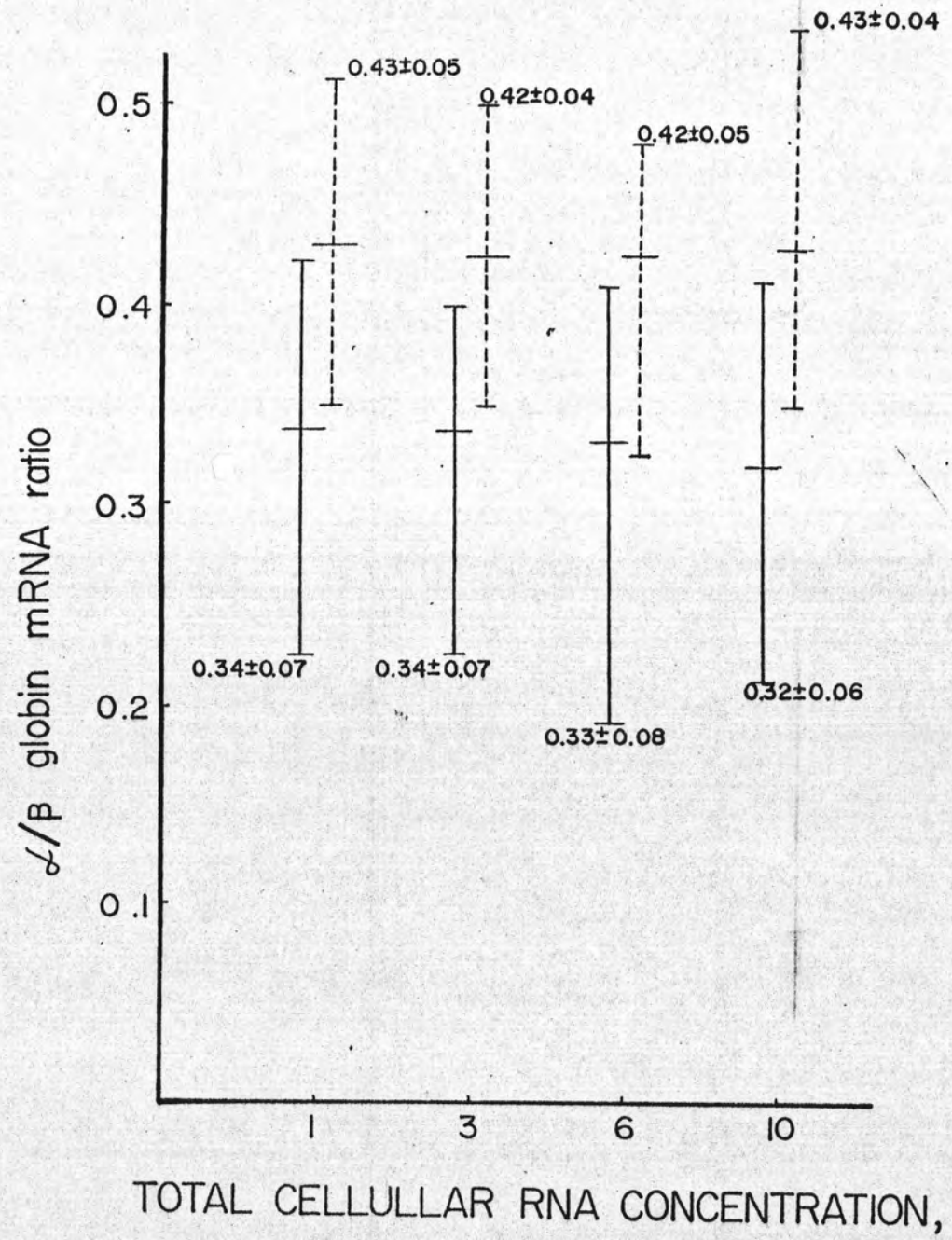


Figure 13. The mean of α/β mRNA ratio in total cellular RNA at various concentration.

——— α-thal$_1$/Hb CS.

----- α-thal$_1$/ α-thal$_2$

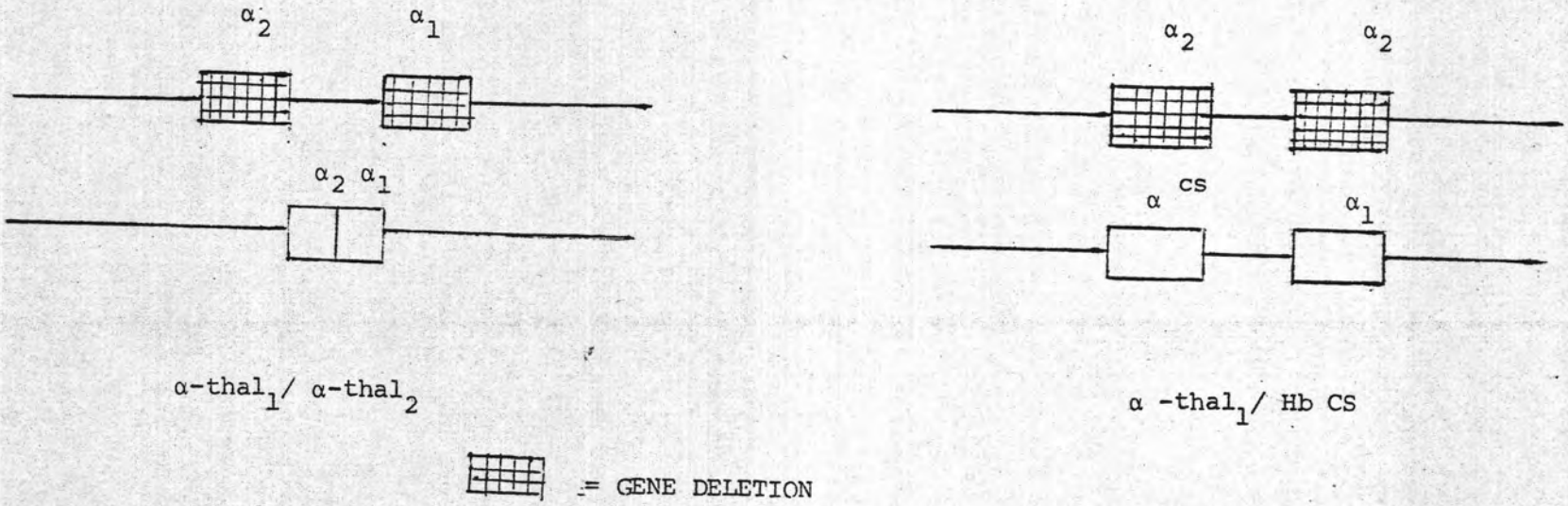


Figure 14. The genotype of Hb H α-thal$_1$/α-thal$_2$ and α-thal$_1$/Hb CS.