

Preparation of A. agile polynucleotide phosphate.AZ AGILE - OCHOA Ia I<sub>2</sub>

3-27-58

From 2 24 hr. agar slants, inoculate 2 150 ml. batches of media. One slant with light growth, one heavy.

5 PM. 30° room. With strong shaking.  
New Brunswick Scientific Co., ~~Rotary~~ Shaker, Model V-3, at 11.5+

3-28-58

9 AM. Abs<sub>500</sub> (Photomultiplier) (water as blank) <sup>UV lamp</sup> <sub>Blue filter - tube.</sub>

	light tube	heavy tube	fresh media	Time	Remarks
Abs <sub>500</sub> =	1.05	1.20	.028	9 AM	
Abs <sub>500</sub> =	<del>1.167</del> 1.350	1.467		11 <sup>35</sup> AM	
Abs <sub>500</sub>	1.40	1.48		1 <sup>35</sup> PM	light yellow.

1.40 PM, Transfer 5 ml of heavy inoculum to new 150 ml. batch of media. Store at 80°C til 5 PM, start at 30° with shaking. Continue original flask.

Abs<sub>500</sub> 1.556 1.632 5 PM  
DISCARDED.

3-29-58

9<sup>20</sup> am - ABS<sub>500</sub> = 1.442

11<sup>50</sup> am 1.542

12<sup>00</sup> - Inoculate 150 ml media with 5.0 ml this culture and hold inoculated media in ice bath.

7<sup>30</sup> pm Warm inoculated media to approx 30° and incub. with shaking as above.

3-30-58

1<sup>15</sup> PM ABS<sub>500</sub> = 1.589

Inoculate 150 ml. media with 5.0 ml. this culture and hold inoculated media in ice bath. In ice bath with shaking as above - Start 1<sup>30</sup> PM

3-31-58

8<sup>45</sup> AM Abs<sub>500</sub> = 1.469

11<sup>40</sup> am Abs<sub>500</sub> = 1.556

Inoculate 150 ml media (1 liter flask) with 5.0 ml this culture. Incub. 30° with shaking. Start 12<sup>00</sup> noon

4-1-58.

8:45 AM, 1.702 (~~Save this in cold room~~),  
Inoculate 150 ml <sup>fresh</sup> medium with 5 ml of this culture. Save  
at 0° until 1:55 PM. Inoculate 300 as above.

4-2-58

8:45 AM, Abs<sub>500</sub> = 1.613.

Inoculate 3 150 ml. batches fresh medium, each  
with 5 ml. of this culture. Save at 0° til 1:30 PM,  
warm to room temp. At 2 PM, start incubating at 30°  
as above.

⊖ Remainder of Abs<sub>500</sub> 1.013, centrifuged, Savall,  
Angle head, 30 min. 110 on rheostat. Accumulate  
all cells in 1 centrifuge tube (122 ml. media  
recovered and discarded.  
Obtained 0.94 gm. (wet weight) cells per 122 ml.  
equivalent to 0.0077 gm/ml. or 7.7 g/liter.  
Store cells in deep freeze.

4-3-58,

Flasks inoculated 4-2-58. Started 2 PM. (Abs<sub>500</sub>)

- |   |                  |       |            |
|---|------------------|-------|------------|
| 1 | <del>1.508</del> | 1.629 | } combine. |
| 2 | 1.708            |       |            |
| 3 | <del>1.645</del> | 1.630 |            |

Inoculate 4 20 liter carboys each containing  
15 liters media. Inoculum = 75 ml per carboy of combined  
cell suspension above. Start at 30° room temp  
at 2 PM - pulling air through  
unused, combined inoculum

Collect cells by centrifugation as above -  
147 ml. media → 0.95 gm cells or 6.5 g/liter.

4-4-58



PREP OF A. AGILE POLYNUCLEOTIDE PHOSPHORYLASE.

4-3-58 - CARBOY CULTURES CONTINUED.

		CARBOY #1	CARBOY #2	CARBOY #3	CARBOY #4
2 <sup>10</sup> pm	air flow - liters/min	15.1	13.5	15.7	12.4
4 <sup>50</sup> pm	" "	15.5	13.2	16.1	12.5

4-4-58

		CARBOY #1	CARBOY #2	CARBOY #3	CARBOY #4
11 am	air flow - liters/min	14.4	12.4	15.2	11.8
11 <sup>30</sup> am	" "	14.9	12.8	15.0	11.4
	<u>O.D. 500</u>	<u>.437</u>	<u>.528</u>	<u>.509</u>	<u>.613</u>

← REMOVE SAMPLES FOR O.D. 500.

No foaming yet but bulbs beginning to be more persistent - particularly #2 - may have to add antifoam later.

1 <sup>15</sup> pm	Add Antifoam	0	ca 0.6ml	0	0
3 <sup>10</sup> pm	Air - liters/min	14.2	12.2	14.4	11.3
	Remove O.D. splees + add antifoam - ca 1.1ml	ca 1.1ml	ca 1.1ml	ca 1.1ml	ca 1.1ml
3 <sup>25</sup> pm	Air - liters/min	14.2	12.2	14.4	11.5
	<u>O.D. 500</u>	<u>.818</u>	<u>.857</u>	<u>.865</u>	<u>.918</u>

5 <sup>15</sup> pm	Air - liters/min	14.2	12.1	14.3	11.3
	<u>O.D. 500</u>	<u>.918</u>	<u>.960</u>	<u>.958</u>	<u>1.005</u>

← TAKE SPLEES FOR O.D. 500 + PUT VACUUM BACK ON.

10 <sup>30</sup> pm	Air - liters/min	13.8	11.9	14.1	11.0
	<u>O.D. 500</u>	<u>1.164</u>	<u>1.160</u>	<u>1.164</u>	<u>1.192</u>

← SPLEES FOR O.D. 500 + VACUUM BACK ON

4-5-58

12 <sup>30</sup> am	Add antifoam	1.0ml	1.0ml	1.0ml	1.0ml
10 <sup>15</sup> am	Air - liters/min	13.5	11.6	13.9	11.0
	<u>O.D. 500</u>	<u>1.427</u>	<u>1.423</u>	<u>1.422</u>	<u>1.432</u>

← REMOVE CULTURES FROM 30° ROOM TO HARVEST.

11<sup>00</sup> am - Began harvesting cells in large refriger sharps.  
 1<sup>00</sup> pm - Had finished running cultures thru sharps. Cells were very well packed and contained very little water. Transfer to tared plastic cent tubes to store in freezer. Tare for balance - 0.35 gm.

Tube #1 = 7.49 → 27.49 = 20 gm cells	#5 = 6.89 → 26.89 = 20 gm	#9 = 7.03 → 27.03 = 20 gm
" #2 = 6.35 → 26.35 = 20 gm "	#6 = 6.96 → 26.96 = 20 gm	#10 = 7.76 → 27.76 = 20 gm
#3 = 7.66 → 27.66 = 20 gm "	#7 = 6.18 → 26.18 = 20 gm	#11 = 6.89 → 14.35 = 7.46 gm
#4 = 8.14 → 28.14 = 20 gm "	#8 = 7.15 → 27.15 = 20 gm	

(OVER)

TOTAL CELLS = 207.5 gm.

About 3 pm - Put cells in freezer.



Preliminary Extract of <sup>Page 3</sup> *A. vinelandii*

Singer  
Hulmoe  
4-4-58

Two batches of cells obtained during preliminary transfer  
(pg 1) 4-2-58. 0.94 gm } pooled. 1.89 gm cells  
4-3-58 0.95 gm }

Homogenize to suspend cells with 1.4 ml. 0.01 N  $KPO_4$  pH 7.4  
Wash into chamber of 10 KC Raytheon Oscillator <sup>(ice water cooling)</sup> so that total  
volume of 0.01 N  $KPO_4$  (pH 7.4) = 11 ml.

Oscillate 10 min. 72 v, 1.1 amp. Remove 4.5 ml. aliquot  
Oscillate 10 min more (20 min) 72 v, 1.1 amp. Collect.

Centrifuge 1 hr. top speed, Servall.

10' sonicate - 4.2 ml extract, 20 min. sonicate - 4.7 ml. [Clear, red brown]

Protein Assay (Microprotein).

	H <sub>2</sub> O	Coleman	- Bl	mg Amt Sample	mg prot ml. extract	Total mg	mg gm cells	
Blank	.50 <del>1.50</del>	.055						
Plasma Albumin St. <sup>2.8 mg/ml</sup> (.02 ml.)	.48	.401	.346	.056				
10 min. Dilute 1-10 (.05 ml.)	.45	.391	.336	.054	10.8	10.6	44.5	.73
" " (.08 ml.)	.42	.565	.510	.083	10.4			
20 min. Dilute 1-10 (.05 ml.)	.45	.409	.354	.057	11.4	11.2	52.6	.81
" " (.08 ml.)	.42	.592	.537	.087	10.9			

Activity Assay. [15 min @ 37°C]

	1	2	3	4	5	6	7	8	
R-v mix.	0.300	.300	.300	—————→ X					
0.01 M $KH_2PO_4$ <sup>Power, 10-29-57. mch</sup>	0.350	.350	.350	—————→ X					
ADP 34 $\mu$ m / ml.	0.075	-	-	.075	—————→ X				
10' extract.	-	.100	-	.100	.030	.010	-	-	
20' extract.	-	-	.100	-	-	-	.100	.030	
H <sub>2</sub> O → V <sub>f</sub> = 100 ml.	0.275	<del>.250</del> .250	.250	.175	.245	.265	.175	.245 X	
cpm. corr. for bleed (see counting book)	4.7	18.6	<del>18.6</del> 33.2	265	195	78	194	100	
cpm / 1 ml incub.	52	245	365	2910	2140	860	2130	1100	
$\mu$ mols P <sub>i</sub> /line.	.009	.042	.063	.504	.370	.149	.368	.190	

Tube	$\mu\text{mP inc.}$	- no enz	- no sub	mg enz	$\frac{\mu\text{mP inc.}}{\text{mg}}$	$\frac{\text{units}}{\text{ml}}$	Total units	units/gm cells
1	.009							
2	.042							
3	.063							
4	.504	.445	.453	1.1	.41	4.3	18	25
5	.370	.361	.319	.33	.97	10.3	43	59
6	.149	.140	.098	.11	.89	9.4	39	53
7	.368	.359	.296	1.1	.27	3.2	15	19
8	.190	.181	.118	.33	.36	4.0	11	23

### Calculations

For total counts - dilute 0.02 ml of TCA sup of #1  $\rightarrow$  2 ml, plate 0.50 ml, ~~200~~ ep 176 cpm corr. for background.

$$\frac{176 \times 2}{.01} = 35,200 \text{ cpm/ml of incubation}$$

$$\text{For cpm/ml of incub} = \frac{\text{cpm/1 ml plated} \times 5 (1.1)}{.5} = \text{cpm/ml plated} \times 11.$$

Background = 24. [Tracer lab - thin window, wet samples used]

$$\mu\text{moles P. inc} = \frac{\text{cpm incorp.} (\mu\text{mP.} + \mu\text{m ADP})}{\text{cpm in P.}} = \frac{\text{cpm inc.} [3.5 + .05 + 2.55]}{35,200}$$

$$= \frac{\text{cpm inc.} \times 6.10}{35,200} = \text{cpm inc.} \times (.000173)$$

### Remarks

Assay Need more than 5 min. centrifugation of TCA solutions, so centrifuge in cold, 10 min. to get clear ~~also~~ solution. Should plate 1 ml of each for constant values.

10<sup>1</sup> extract better than 20<sup>1</sup> s.a. Evidently tube #4 had too high an activity to be linear. From 5-6, s.a. is about what reported in tables but not as high as in text of prepent. Similarly, 50-60 units/gm of cells obtained compares with prepent value of 75-100.



7 April '58

118 ml / 20g cells

Harvested cells placed in 10 tubes containing 20 gm. each and an 11<sup>th</sup> containing 7.46 gm. Contents of each of first 10 tubes gently homogenized with small volume (2-15 M/100 KPO<sub>4</sub> buffer pH 7.4 and made up to 118 ml. <sup>-20%</sup>

~~The 11<sup>th</sup>~~ tube made up to 44 ml. with buffer and treated similarly. Contents of each tube sonicated separately in 10 KC Raytheon oscillator current 1.1 amp. and following voltage settings:

# 1	82	# 7	82
2	?	8	83
3	86	7	84
4	87	10	82
5	89	11	82
6	84		

Sonicates in ~ 30 ml portions (total of 33 tubes) centrifuged 1 hour at 20,000 x g in Small. Total volume supernatant = 1043 ml. -

Remove 2.0 ml sple = 4-7-AR58

1041 ml. 1M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. Add 5.2 ml 0.2 M Versene soln. Weigh out 257 gm (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. pH before addition of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (20° soln. aliquot diluted 1:5 with cold distilled H<sub>2</sub>O) gives 6.3. Add (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> over 45 min. period. Adjust pH with 1M KOH to keep ~ 7.0.

Total KOH added in course of pptn. = 6.2 ml.

After 30 min. <sup>additional stirring</sup> pH drops from ~~7.12~~ 7.12 to 6.6. Add 1 ml KOH, raise pH (of diluted aliquot) to 7.3. Undiluted pH = 7.38

Centrifuge for 60 min. in 32 centrifuge tubes 20,000 x g. Supernatant collected in 2 ~~liter~~ liter beaker (clear dark brown soln.) and 81.6 gm. (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> added at 0° (40 min)

Note:  
all seawall  
run  
Full  
Speed  
≡  
"20,000 x g"

7 April '58

pH after addition of 3 ml KOH 1M is  $\approx 7.6$ . Material diluted 1:5 has pH = 7.42 at end. Stir for additional 30 min., centrifuge in 32 tubes at 20,000 x g for 50 min. Collect ppt. in about 30 ml 0.01 M  $PO_4$  pH 7.4, in which all dissolved to form dark brown soln. Dialyzed in rocking apparatus overnight vs. 0.033 M succinate pH 6.3 to 12 liters of which were added 0.946 gr. cysteine hydrochloride of P, to give  $5 \times 10^{-4}$  M/l. Dialysis started 11:30 PM rate 860 ml/hr.

4-8-58

Stopped dialysis 100 gm - Volume = 50.2 ml. = 4-7-BR<sub>58</sub>. Very dark brown viscous solution. Had used 9-10 liters of succinate.

$\frac{d_{280}}{d_{260}}$  RATIOS:

	DILUTION IN H <sub>2</sub> O	$\frac{d_{280}}{d_{260}}$	$\frac{d_{260}}{d_{260}}$	$\frac{d_{280}}{d_{260}}$
4-7-AR (SONIC EXTRACT)	1:10 (0.5 + 4.5)	> 3.0	2.51	
" "	1:50 ( $\frac{11.50}{0.6} \times 1:10$ ) (0.6 ml 1:10 + 2.4 ml H <sub>2</sub> O)	.883	1.402	0.630 } 0.633
" "	1:75 ( $\frac{11.75}{0.6} \times 1:10$ ) (0.6 ml 1:10 + 2.4 ml H <sub>2</sub> O)	.598	.942	
4-7-BR (DIAL. AS. FRACT)	1:100 (0.1 ml $\rightarrow$ 10.0 ml)	1.902	2.60	0.731
" "	1:500 (0.6 ml 1:100 + 2.4 ml)	.418	.588	0.711 } 0.713
" "	1:600 (0.5 ml 1:100 + 2.5 ml)	.344	.482	



Protein Determinations

4-7-58

Extract. [1043 ml.]

10.430 gm protein, total.

Sample	ml. sample	ml H <sub>2</sub> O	col. min	-Bl	mg/sample/ml	mg/ml
Blank	—	0.15	.058			
Stand. <sup>2.5 mg/ml</sup> <sub>plasma</sub>	.02	0.48	.396	.338	.056	
Extr. dil-10	.05	0.45	.365	.307	.081	10.2
	.08	0.42	.509	.451	.075	4.4
	.02	0.48	.145	.127	.021	10.5

} 10.0

Total counts/inc. = 30,600 cpm.  
Background = 24.5 (Tracer blank)

Activity Assay

	1	2	3	4	5	7	8	6
P-Y mix	0.300	0.300	0.300	0.300	0.300	0.300	0.300	0.300 X
0.01N KH <sub>2</sub> PO <sub>4</sub> <sub>DuPont 10-29-57</sub>	0.350	0.350	0.350	0.350	0.350	0.350	0.350	0.350 X
ADP <sup>3 μmole</sup> <sub>1 μmole</sub>	0.075	0.075	0.075	0.075	0.075	<del>0.075</del>	<del>0.075</del>	—
Extract	—	0.100	0.050	0.020	0.010	0.100	0.020	0.050
H <sub>2</sub> O to vol. 1.000 <sub>(see bench)</sub>	0.275	0.175	0.225	0.255	0.265	0.250	0.330	0.300
cpm. corr. for blank	7.2	365	255	177	116	148	0.0	5.5
cpm / 1 ml. incub. <sub>inc.</sub>	79	4010	2810	1950	1280	218	—	605
μmoles P <sub>i</sub> / inc. <sub>inc.</sub>	<del>798</del>	1.798	.559	.388	.255	.043	—	.012
- no. eoz.		.782	.543	.372	.239			
- no. sub.		.739	.531	.372	.239			
mg. eoz.		1.0	0.5	0.2	0.1			
mg P <sub>i</sub> / gm / gm / gm		.739	1.06	1.86	2.39			
Total units =				19500	25000			
units / gm cells				94	120			

cpm. inc. 1.05 + 2.55 = 3.60  
1 pm<sup>3</sup> / inc.





Zn-ETOH Fractionation

A. vinelandii  
Polynucleotide Phosphorylase

Solutions

0.1 M Zinc acetate

Diluting

0.033 M succinate, pH 6.3 (0.0005 M cysteine)  
[66 ml. 0.25 M → 500 ml. Add before using 0.040 gm.  
cysteine-HCl·H<sub>2</sub>O].

0.0005 M

Take-up

0.1 M KPO<sub>4</sub>, pH 7.4 & 0.03 M Versene,  
[add cysteine-HCl (0.176 gm/100 ml) check pH make  
to 100 ml before using]

0.03 M  
0.176

Dialyze

0.01 M KPO<sub>4</sub>, pH 7.4, 0.001 M in Versene,  
4.22-58

48 ml. 4-7-BR, Am Sulf 64 mg/ml. = 3070 mg.

Dilute to 307.0 ml. & diluting buffer.

ETOH to 15% by volume. 56 ml. Abs. ETOH.

0.1 M ZnAc → 0.004 M Total = 363.0

377.5

1450 μmole = 14.5 ml.

Start 11<sup>55</sup> AM. To add 56 ml Abs ETOH. & bath Temp set to  
-1/2 < 1

Finish adding 11<sup>55</sup> AM. Str. at -5°C hl. -2/2 -4.5

12<sup>30</sup> - centrifuge. Servall, Angle Head, Top speed, 1 hour.

In cold rm at -15.5°C. Heads cooled at 0°, 1 hr

10 min at -15.5°C. Final soln. temp. after centrifugation  
-7.5°C

Total volume should be 363 + 14.5 = 377.5 ml.

ETOH to 20% by vol. 38 ml.  $\frac{94}{415.5} \sim 22\%$

ZnAc → 0.014 M, disregarding previous Zn.  $\frac{94}{415} \cdot 20\%$   
(0.1 M) = 4% (0.014). 59 ml.

Start 2:12 PM at -5° Finish 3:55 PM at -9°

Transfer to bath ~ -90, move to cold room and centrifuge Seewall  
 1 hour top speed. Room temp. rises to ~~room~~ -5°C during  
 centrifugation. [treads cooled before centrifugation for 30 min.]  
 Temp. of supernatant at end of ~~10000~~ centrifugation = +9°C.]

Take up protein of both fractions in min. volume of take-up  
 solution. Fr. I dissolved completely but Fraction II was  
 more difficult and was a suspension. Each was dialyzed  
 against dialysis solution, rocking dialyzer, running, ~~distilled~~  
 solution, 4°C, starting at 5:30 PM.

4-23-58

11 AM Stop dialysis. Fr. I, 70 ml. 4-22-RCR 58  
 Fr. II 44 ml. 4-22 DR 58.

PROTEINS

	Sample	4.0	0.0.	-BI	mg/sample	mg/ml	Total mg
Blank		.500	.071	-			
Stand Plasma Alb. 2.8 mg/ml.	0.020	.480	.423	.352	.056		
I dilute 1-100	0.050	.450	.147	.076	.012	24	22.5 = 1570mg
	0.200	.300	.334	.263	.042	21	
II, dilute 1-50	0.200	.300	.668	.597	.095	24	= 1060mg
	0.500	-	>1.5				

See pg. 10 for repeat on  
 Fr. II



4-23-58.

Activity Assay on A. Agile Prep

New R-Mix

✓ 3 ml. 1 M Tris, pH 8.2

✓ 1.5 ml 0.1 M MgCl<sub>2</sub>

✓ 0.6 ml .05 M Versene

✓ 0.3 ml P<sub>i</sub><sup>32</sup>, 4-22-58, 5.6  $\mu\text{m Pi}/\text{ml}$ .  $\sim 8.43 \times 10^6 \text{ cpm}/\mu\text{M}$  (Tracerlab)

✓ 3.6 H<sub>2</sub>O

9.00

V<sub>F</sub> (.19  $\mu\text{m Di}/\text{ml}$ )

Activity Assay P

		1	2	3	4	5	6	7	8	9	10
R-Mix	X	.300	.300	.300	.300	.300	.300	.300	.300	.300	.300
0.01N K <sub>2</sub> HPO <sub>4</sub>	X	.350	.350	.350	.350	.350	.350	.350	.350	.350	.350
ADP 34 $\mu\text{m}/\text{ml}$ Dowex 10-29-57 del 1-100	X	.075	.075	—	.075	.075	.075	<del>0.075</del>	.075	.075	—
AmSulf 4-7-BR <sub>58</sub> (64 mg/l)		—	.050	.050	—	—	—	—	—	—	—
Zn-ETOH I 4-22-OR <sub>58</sub> 22.5 $\mu\text{g}/\text{ml}$		—	—	—	—	—	—	—	—	—	—
Zn-ETOH II 4-22-OR <sub>58</sub> 24 $\mu\text{g}/\text{ml}$ del 1-100		—	—	—	.100 <sup>X</sup>	.050	.200	.100	.200	.100	—
H <sub>2</sub> O to V <sub>F</sub> = 1.00 ml	X	.275	.225	.300	.175	.225	.075	.250	.075	.175	.150
cpm - bkgnd = 19.9		12.9	282	157	577	148	655	16.1	870	722	70.6
- no enzyme			269		564	135	642		857	709	
- no substrate			253			119	<del>636</del> 626		786	638	
cpm / 1 ml incub.			2780			1310	6880		8650	7020	
Total $\mu\text{m Pi}$ + ADP		2.5 $\mu\text{m ADP}$ + 3.5 $\mu\text{m Pi}$ + 1.06 $\mu\text{m Pi}$ = 6.06 $\mu\text{m Pi}$									
Total cpm		12 x 10 <sup>4</sup>									
s.a. P <sup>32</sup>		1.98 x 10 <sup>4</sup> cpm / $\mu\text{mole}$									
$\mu\text{mole Pi}$ exch.		.140			.066	.348		.437	.354		
s.a.		4.4			5.5	7.3		11.0	11.5		
Total units		4.4 (30%) 13,500			5830	7750		1570	2360		

See pg 10 for repeated assays

Assays on Zn-ETOH Fractions (cont)  
REPEATS

Protein on Fraction II.

	Sample	H <sub>2</sub> O	OD	-Bl	mg/sample	mg/ml	Total mg
Blank	—	.500	.062				
Standard, Plasma Albumin, 2.8mg/μl.	.020	.480	.399	.337	.056		
4-22-DR <sub>58</sub> , dilute 1-100	.100	.400	.242	.180	.030	30	} 29 1275
	.200	.300	.397	.335	.056	28	

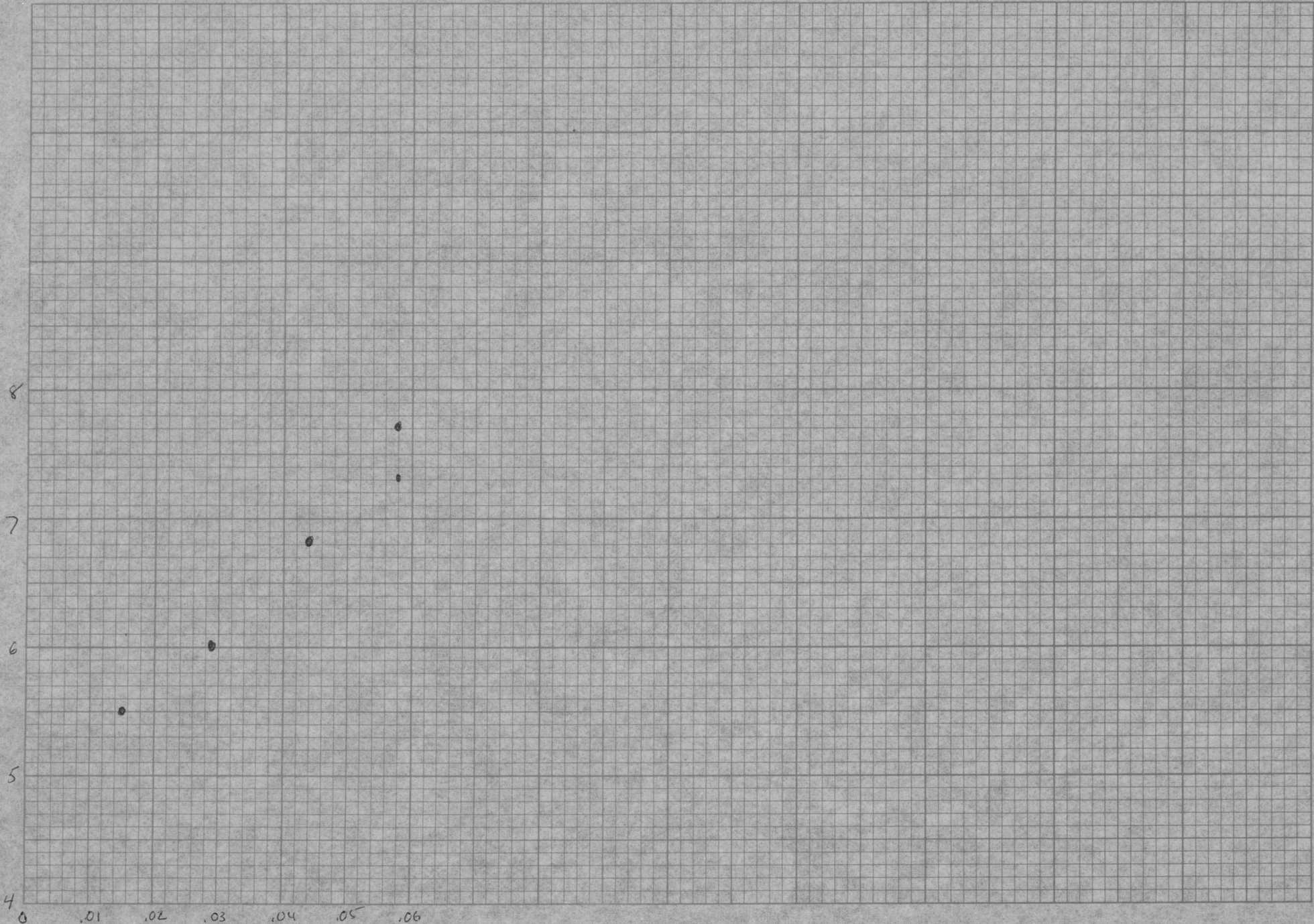
% of protein of Am.Sulf.  $\frac{1275}{3076} = 42\%$  in Fr. II.

$\frac{1570}{3270} = 51\%$  in Fraction I.

Activity (enzyme diluted in 0.05M Tris, pH 8.2)

	1	2	3	4	5	6	7	8
R-Mix (4-23-58)	X .300	.300	.300	.300	.300	.300	.300	.300
0.01N KH <sub>2</sub> PO <sub>4</sub> Dowex 16-29-57	X .350	.350	.350	.350	.350	.350	.350	.350
ADP 34 μM/ml. 22.5mg/ml Dil 1-10	X .075	.075	.075	—	.075	.075	.075	—
Zn-ETOH I, 4-22-CR 29mg/μl Dil 1-100	X —	.080	.040	.080	—	—	—	—
Zn-ETOH II 4-22-DR	X —	—	—	—	.200	.150	.100	.200
H <sub>2</sub> O to V <sub>f</sub> = 1.00ml.	X .275	.195	.235	.270	.075	.125	.175	.150
mg. enzyme		.184	.092	.184	.058	.044	.029	.058
Total μm Pi + ADP =	2.5 (ADP) + 3.5 (Pi) + 0.06 (Pi) = 6.06 μm.							
Total cpm / incub.	10.3 x 10 <sup>4</sup>							
s.a. P <sup>32</sup> .	1.70 x 10 <sup>4</sup>							
cpm - bkgd = 2217	2.5	421	228	23.8	712	478	286	14.7
- no enz.	—	418	225		709	475	283	
- no sub.		394	201		694	460	268	
cpm / ml inc. μmoles exchanged		4330	2210		7640	5060	2950	
μmoles exchanged s.a.		.254	.130		.449	.298	.174	
		1.4	1.4		7.7	6.8	6.0	
Total units		2200			9810	8650	7650	
% of units in Am. Sulf.		16%			72.5	64	57%	





mg / ml. SA.

4-22 DR<sub>58</sub> (2n-ETOH II)

Centrifuge 1 hour, top speed, Servall, Angle Head.  
to try to remove precipitate. Small precipitate collected,  
clear supernatant fluid. Save ppt frozen. Save supernatant frozen.  
(4-22-DR<sub>58</sub>-Pee)

Activity Assay

	1	2	3	4	5	6
R-Mix (4-23-58)	.300	.300	.300	.300	.300	.300 ✓
0.01 N KH <sub>2</sub> PO <sub>4</sub> Dowex 10-24-57	.350	.350	.350	.350	.350	.350 ✓
ADP 34 μm/M del 1-100	.075	.075	.075	<del>.075</del>	.075	— ✓
Centr. 4-22 DR <sub>58</sub> 28 mg/ml	—	.100	.200	.100	—	—
4-22 DR <sub>58</sub> 29 mg/ml del 1-100	—	—	—	—	.200	.200 (see pg 12 for prot) ↑
H <sub>2</sub> O & V <sub>F</sub> = 1.00	.275	.175	.075	.250	.075	.150 ✓
cpm - bkgd = 21.1	6.1	205	415	15.1	474	<del>317</del> 10.6
- no enz.		199	409		468	
- no sub.		184	394		<del>457</del> 456	
Total cpm incorp		2020	4330		<del>4800</del> 5020	
cpm / incub		81,400				
P <sub>i</sub> / incub		81,400	6.06			
s.a. P <sub>i</sub> <sup>32</sup>		<del>6.06</del>		13,400	(1.34 × 10 <sup>4</sup> )	
μmoles P <sub>i</sub> / incorp / incub.		.151	.323		<del>.375</del> .356	
mg. prot / incub.		.028	.056	.028	.058	.058
μmoles P <sub>i</sub> / incub / mg		<del>.54</del> .609	5.8		6.5	

280/260 ratio - Dilute 1-300 (.01 → 3.00) (H<sub>2</sub>O)

	d <sub>280</sub>	d <sub>260</sub>	$\frac{280}{260}$	F <sup>(w/c)</sup> mg/ml	μmol mg/μ
4-22-DR <sub>58</sub>	.252	.347	.73	.508	.128
4-22-DR <sub>58</sub> centrifuged.	.239	.333	.72	.49	.117



Protein on centrifuged 4-22-DR<sub>58</sub>.

	Sample	H <sub>2</sub> O	O.D.	-Bl	mg/sample	mg/ml
Blank	—	.500	.063	—		
Standard Plasma Alb. 2.8 mg/ml	0.020	.480	not done, use 4-24 figure.			
4-22-DR <sub>58</sub> (centr.) dil 1-100	0.100	.400	.239	.176	.029	29
	0.200	.300	.377	.314	.052	26

New R-mix

3 ml. 1 M Tris, pH 8.2, 1.5 ml 0.1 M MgCl<sub>2</sub>, 0.6 ml 0.05 M Verseine  
 0.6 ml P<sub>i</sub><sup>32</sup> (4-22-56, 5.6 μM P<sub>i</sub>/ml) + 3.3 M H<sub>2</sub>O → 9.0 ml total

For Assays 0.300 ml R-mix, 0.34 ml P<sub>i</sub> (KH<sub>2</sub>PO<sub>4</sub>, 0.01N); 0.075 ml ADP  
 (34 μM/ml) enzyme, H<sub>2</sub>O → 1.00 ml. [Vol-Enz, H<sub>2</sub>O =  $\frac{.715}{.60}$ ]

(centr) 4-22-DR<sub>58</sub> 2n-ETOH II  
 28 mg/ml. 0.8 = 22.4 mg.

dilute to 2.24 ml. by adding 1.44 ml 0.01N KPO<sub>4</sub>, pH 7.4 @ 0.18 M.  
 of 4-22-DR<sub>58</sub>, centrifuged.

Add .035 ml 1N HAc to pH ~ 5.5 (chlorophenol red). Some precip. formed.  
 Add ~~0.25 ml~~ 0.24 ml Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> gel (Sigma lot 116-240) [22.8 mg/ml]  
(over 3 ml)  
 slowly, cold. Shake 20 min. Spin 10 min. ~~26~~ 13,000 rpm, 21,600 x g  
 (Lourdes).

Sup I = 2.2 ml. Remove 0.2 ml aliquot.  
 Ppt I - susp in 0.3 ml 0.1 M KHPO<sub>4</sub> pH 6.  
 Add 0.48 ml gel as above, centrifuge.  
 Sup II = 2.4 ml  
 Ppt II - susp. in 0.3 ml 0.1 M KHPO<sub>4</sub> pH 6

III Repeat gel step as in #II. (0.48 ml gel).

Sup III = E<sub>3</sub> sup - pH ~ 5.7 remove 20 ml dple. - TOTAL VOL = 2.6 ml.

pp't III - ADD 0.3 ml 0.114 KPO<sub>4</sub> pH 6.0 TO ELUTE. CENT AFTER ca 30 MIN.

IV Repeat, (0.48 ml. gel). Let stand 31 min. before centrifuging.  
2.7 ml. final supernatant.

pp't IV - <sup>(eluted)</sup> treated as above. (15 min.) then centrifuged.

all eluates and samples of supernatants stored frozen.

All supernatants contained some precipitate. Even the final one was not clear.





Helmoe  
Singer  
5-5-58

page 14

Assays on Preliminary  $\text{Ca}_3(\text{PO}_4)_2$  gel step

	1	2	3	4	5	6
H <sub>2</sub> O (V <sub>f</sub> = 1.00)	0.160	<del>0.145</del> 0.085	0.085	0.160	0.085	0.160 <sup>✓</sup>
R-Mix (5-5-58)	0.300	0.300	0.300	0.300	.300	.300 <sup>×</sup>
KH <sub>2</sub> PO <sub>4</sub> (0.01M)	0.340	0.340	0.340	0.340	.340	.340 <sup>×</sup>
ADP 34 μm/ml dil 1-100	-	0.075	0.075	<del>0.075</del>	.075	- <sup>×</sup>
4-22 DP <sub>55</sub> centr. dil 1-25	0.200	0.200	-	-	-	- <sup>×</sup> 0.01 → 1.00 (0.99 ml P <sub>u</sub> , 0.01 ml)
gel sup I dil 1-25			0.200	0.200	-	- <sup>×</sup> (0.02 → .50 (0.48 ml P <sub>u</sub> , 0.5 ml)
gel sup II					.200	.200 <sup>×</sup> (0.02 → .50 " " )
cpm-blkqd	1613	606	278	<del>145</del> 1815	115	16.7
- blank	<del>1613</del>	590	259		98	
Total cpm/inc. incorp.		6480	2850		1080	
Total cpm/inc		114,000 cpm.				
Total P		2.5 (ADP) + 3.4 (P <sub>i</sub> ) + 0.1 (P <sup>32</sup> ) =			6.00	
s.a. P <sup>32</sup>		19,000 cpm/μmole				
μmoles P <sup>32</sup> exch.		.341	0.150	<del>0.057</del>	0.057	
μmoles P <sup>32</sup> exch / ml enz		171	18.7		7.1	
μm P <sup>32</sup> exch / mg enz		611	211		171	
Total units		1370	41		17	
		22.4 mg used				

Inorganic P<sub>i</sub> det'm on TCA sup. (0.05 ml aliquot) (Fiske Sub)

	O.D.	-Bl	AMP <sub>i</sub>	AMP <sub>i</sub> /inc.
Blank stand	.032	-	-	
1	.251 .206	.219	0.200	3.50
2	.214	.182	.166	3.65
3	.205	.173	.158	3.48
4	.205	.173	.158	3.48
5	.216	.184	.168	3.70
6	.211	.179	.163	3.59



Assays on Preliminary  $\text{Ca}_3(\text{PO}_4)_2$  Gel Step (cont)

	1	2	3	4	5	6	7	del. $\bar{c}$ 0.05 M Tris, pH 8.2.
R-Mix (5-58)	0.300	—————						X
$\text{KH}_2\text{PO}_4$ (0.01N)	0.340	—————						X
ADP 34 $\mu\text{m}/\text{ml}$	0.075	—————						X
Eluate I <sup>del 1-50</sup>	0.200	—————						(0.01 $\rightarrow$ 0.50) (0.49) ✓
Eluate II 1-25	—	0.200	—————				—	(0.02 $\rightarrow$ 0.50) (0.48) ✓
Eluate III 1-10	—	0.200		—————			—	(0.05 $\rightarrow$ 0.50) (0.45) ✓
Eluate IV 1-10	—	0.200			—————		—	(0.05 $\rightarrow$ 0.50) (0.45) ✓
Sup III 1-20	—	0.200				—————		(0.02 $\rightarrow$ 0.40) [0.38] ✓
Sup IV <del>1-5</del>	—	0.200				—————		(0.10 $\rightarrow$ 0.50) [0.40] ✓
$\text{H}_2\text{O}$ to $V_f = 1.00$	.085	.085	.085	.085	.085	.085	.285 <del>.760</del>	X
cpm - bkgd = 22.0	409	755	49.5	23.0	12.6	47.2	1.2	
- Blank	408	754	48.3	22.8	11.4	46.0		
Total cpm incorp.	4490	8290	532	240	125	506		
Total counts,	112,000 cpm / incubation.							
Total $\text{P}^{32}$ phosph.	= 6.00							
$\text{P}^{32}$ , s.a. =	18,700 cpm / $\mu\text{mole}$							
$\mu\text{moles } \text{P}^{32}$ exch.	.240	1.443	.028	.013	.007	.027		
$\mu\text{moles } \text{P}^{32}$ exch / ml en.	60	55	1.4	0.7	0.7	0.7		
$\mu\text{moles } \text{P}^{32}$ exch / mg en.	5	9.5	1.88	.8	.2	.7		
Total Units	18	1612	144	124	1.9	2.0		

Proteins on Fractions from Preliminary Gel

Sample	Sample	H <sub>2</sub> O	OD	-Bl	mg prot	mg/ml diluted	mg/ml undil	Total vol. ml.	Total prot.
Bl.	-	0.500	.065	-	-	-	-	-	-
Stand. Pl. A1b 2.8 mg/l.	0.020	0.480	.1410	.345	.056	-	-	-	-
Sup I del 1-25	0.075	0.425	.225	.160	.026	0.35	8.8	2.2	19.4
Sup II del 1-25	0.075	.425	.248	.183	.030	0.40	10.0	2.4	24
Sup III del 1-20	0.100	0.400	.176	.111	.018	0.18	3.6	2.6	9.4
Sup IV del 1-5	0.200	0.300	.322	.257	.042	0.21	1.05	2.7	2.8
Eluate I del 1-50	0.200 <sup>100</sup> <del>0.200</del>	0.300 <sup>2</sup> <del>0.300</del>	.338	.273	.044	0.24	<del>1.2</del> <sup>0</sup>	0.3	3.6
Eluate II del 1-25	0.200	"	.350	.285	.046	0.23	5.8	0.3	1.7
" III del 1-10	0.200	"	.258	.193	.031	0.16	1.6	0.3	.5
" IV del 1-10	0.200	"	.182	.117	.019	0.09	0.9	0.3	.3

Used 0.8 ml of 4-22 DR 58 centr. = 22.4 mg. prot = 137 u.

Sup I + Eluate I = 41 + 18 = 59 units, = ~~23 mg prot~~ [19.4 + 3.6 = 23 mg.]

Sup II + Eluate II = 17 + 16 = 33 units = 24 + 1.7 = 25.7 mg.

Sup III + Eluate III = 1.9 + .44 = 2.3 units = 9.4 + 0.5 = 9.9 mg.

Sup IV + Eluate IV = 2.0 + 0.2 = 2.2 units = 2.8 + 0.3 = 3.1 mg.



TUESDAY 6 MAY 1958.

1M ACETATE BUFFER.

250 ml 2M Sodium Acetate 11-30-56 + 250 ml H<sub>2</sub>O - pH = 6.48 (24°). Add 1N HAc to adj. pH.

1N HAc - 1.0 ml	- pH = 6.43
1.0 ml	6.38
2.0 ml	6.31
3.0 ml	6.21
3.0 ml	6.13
3.0 ml	6.03
5.0 ml	5.98
5.0 ml	5.88
5.0 ml	5.82
15.0 ml	5.68
<hr/>	
45.0 ml	

← remove 4ml for indicator buffer (at 6.21)  
 ← remove 4ml for indicator buffer (at 6.03)  
 ← remove 4ml for indicator buffer (at 5.82)  
 ← remove 4ml for indicator buffer (at 5.68)

250ml above mixture (pH 5.68).

+ 200ml 1N HAc - pH = 4.69 (24°)

10ml	"	4.67 (24°)
<hr/>		
210ml		

= 1M Sodium Acetate <sup>Buffer,</sup> pH 4.67 (24°).

5-7-58 - Remainder of solution above at pH 5.68

+ 1N HAc - 15 ml - pH = 5.48 (24°)

15 ml	5.31
10 ml	5.24
10 ml	5.20
<hr/>	
50 ml	

= 1M Sodium Acetate Buffer pH 5.2 (24°)

2<sup>nd</sup> preliminary gel step.0.8 ml. 4-22-DR<sub>58</sub> (centr.) Zn-ETOH II.28 mg/ml, 0.8 ml = ~~226~~ 22.4 mg.dilute to 2.24 ml  $\bar{c}$  1.44 ml 0.01 N KPO<sub>4</sub>, pH 7.4+ 0.05 ml 1 M Ac buffer, pH 4.67  $\rightarrow$  pH 5.9 - Brom cresol purple  
0.01 ml " " " " " " " " " " " "

solution opalescent, but no real precipitate.

I Add 0.2 ml Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> gel (see 5-5-58). (~~as 5-5-~~  
(4 min.)  
Adding gel + stirring in ice - total time 20 min.)  
Centrifuge as 5-5-58.

Sup I = 2.1 ml, 0.02 ml removed - frozen. - pH ~ 5.7 - BCP

Gel I - elute  $\bar{c}$  0.3 ml 0.1 M KPO<sub>4</sub>, pH 6.0. [5 min @ 1 m. temp, ~~20~~<sup>20</sup> min. on ice,  
<sup>elute Ia.</sup> centrifuge, decant]. Repeat elution as above  $\bar{c}$  pH 7.4 buffer - elute Ib.]II - Sup I + 0.52 ml Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> gel - 6 min, let sit in ice  $\bar{c}$  occurs mix to total 20 min.

Sup II = 2.4 ml, 0.02 ml removed, frozen, pH ~ 5.7

Gel II - elute twice, as above, with 0.1 M KPO<sub>4</sub>, pH 6.0, pH 7.4.III - Sup. II + 0.48 ml Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> gel - 4 $\frac{1}{2}$  min, let sit as above + cent.Sup III = 2.7 ml. ~~to~~ saved frozen. pH ~ 5.8

Get Gel III, elute twice, as above.

Store all fractions frozen.



Singer  
Hilmae  
5-7-58

Assays on second Gel Preliminary.

	1	2	3	4	5	6	7	8	9	10	dilc 0.05M		
R-Mix-5-5-58	0.300	—————→									X	0.82	
KH <sub>2</sub> PO <sub>4</sub> (0.01N)	0.340	—————→									X		
ADP <sub>34</sub> μm/ml	0.075	—————→									X		
Sup I dil 1-50	0.200											0.2 → 1.5 √(1.98)	
Sup II dil 1-20		0.200											0.2 → 1.4 √(1.38)
Sup III dil 1-5			0.200									1 → 1.5 √(1.40)	
Elate Ia, dil 1-50				0.200							0.2 → 1.4 √(1.98)		
Ib dil 1-50					0.200						0.2 → 1.1 √(1.98)		
IIa 1-50						0.200					102 → 1 √(1.98)		
IIb 1-50							0.200				102 → 1 √(1.98)		
IIIa 1-5								0.200			11 → 1.5 √(1.4)		
IIIb 1-5									0.200			1 → 1.5 √(1.4)	

H <sub>2</sub> O & VA = 1.004	0.085	0.085	0.085	0.085	0.085	0.085	0.085	0.085	0.085	0.085	0.285 X
cpm - bkgd = 25.3	<del>227</del>	101	107	132	26.1	915	441	878	915	11.1	
- Control	216	90	96	121	15.0	904	430	867	904		
Total cpm incorp.	2380	999	1060	1330	165	9950	4720	9540	9950		

Total cpm/incubation = 100,000

Total P/incub = 3.4 + 2.5 + 0.1 = 6.0

s.a. P<sup>32</sup> = 16,700 cpm/μmole

μmoles P <sup>32</sup> incorp/μl	142	0.059	<del>1.07</del> <sup>0.063</sup>	0.080	0.010	0.595	0.283	0.571	0.571	1595
μmoles / ml. enz.	355	5.9	1.6	20	2.5	149	70.4	14.3	14.9	
μmoles / mg. prot.	2.7	1.1	0.6	2.6	0.4	17.1	7.1	4.8	3.8	
Total units	75	14.3	0.4	6.0	0.8	44	21	4	5	

used 0.8 ml 4-22-DR<sub>58</sub> = 22.4 mg prot + 137U.

Sup I + El Ia + Ib = 75 + ~~4.3~~<sup>6.0</sup> + 1.8 = 81.8 units, 32.1 mg (27.2 + 2.3 + 2.1)  
 Sup II + El IIa + IIb = 14.3 + 44 + 21 = 79.3 units, 18.6 mg (13.0 + 2.6 + 3.0)  
 Sup III + El IIIa + IIIb = 6.0 + 4 + 5 = 15 units, 9.4 mg (7.3 + 1.9 + 1.2)

40% of units lost.

SINGER  
HILMOE

A2. ENZ. PREP. (CONT.) (I) PROTEINS.

(II) EFFECT OF pH ADJUST. IN GEL STEP.

I Proteins - Phenol - Gel fractions of 5-6-58.

	DILUTION	Sple	H <sub>2</sub> O	OD <sub>660</sub>	rel. O.D.	mg/plate	mg/ml	TOT. PROT. mg.
①	Sup I	1:50	.40 ✓	.228 <sup>2.42</sup>	.158	.0263	13.20	27.7
②	Sup II	1:20	.14 ✓	.299 <sup>3.18</sup>	.229	.0381	5.4	13.0
③	Sup III	1:5	.10 ✓	.394 <sup>4.11</sup>	.324	.0538	2.7	7.3
④	Eluate Ia	1:50	.20 ✓	.257 <sup>2.71</sup>	.187	.0311	7.8	2.3
⑤	" Ib	1:50	.20 ✓	.237 <sup>2.51</sup>	.167	.0278	7.0	2.1
⑥	" IIa	1:50	.20 ✓	.278 <sup>2.92</sup>	.208	.0346	8.7	2.6
⑦	" IIb	1:50	.20 ✓	.310 <sup>3.28</sup>	.240	.0399	10.0	3.0
⑧	" IIIa	1:5	.05 ✓	.253 <sup>2.63</sup>	.183	.0304	3.0	0.9
⑨	" IIIb	1:5	.05 ✓	.302 <sup>3.12</sup>	.232	.0386	3.9	1.2
⑩	Std. PL. ALB 2.8 mg/ml	-	.02 ✓	.399 <sup>4.10</sup>	.329	} .337 = .056 mg.		
⑪	"	-	.01 ✓	.242 <sup>2.50</sup>	.172			
⑫	Blank	-	.50	.061 <sup>0.60</sup>	} .070			
⑬	Blank	-	.50	.078 <sup>0.80</sup>				

USED 0.8 ml 4-22-DR<sub>58</sub> = 22.4 mg prots + 137 U.

II - 0.8 ml 4-22-DR<sub>58</sub> (3m-EnOH II) stirring with rod on motor, add 1.44 ml .01M KPO<sub>4</sub> pH 7.4 + mix.

(a) Remove 1.1 ml diluted enzyme and add 1M acetate pH 4.67 (2x) 5-6-58 to adj to pH ~ 5.6-5.7 - 0.02 ml - pH ~ 5.8-6.0 + 0.05 ml - pH ~ 5.6-5.7. = adjusted 4.67 buff.

(b) Remainder of diluted enzyme - add 1M acetate pH 5.2 (2x) 5-7-58 - 0.02 ml - pH > 6.2; .02 ml - pH ~ 6.1; .02 ml - pH ~ 5.8; 0.005 ml - pH ~ 5.6-5.7  
Total buffer = 0.065 ml. Soln = adjusted to pH 5.2

This was stirred with rod on motor while adjusting pH - in ice.



Singal  
Helmer  
5-7-58.

p. 20.

Assays of pH adjust. experiment.

	1	2	3	4	5		
R-Mu (5-55r)	0.300	0.300	0.300	0.300	0.300		
KH <sub>2</sub> PO <sub>4</sub> (0.01M)	0.340	0.340	0.340	0.340	0.340X		
ADP 34 μm/ml	0.075	0.075	0.075	0.075	0.075X		
concr. 4-22-DR <sub>58</sub> 1-100	0.200	—	—	—	—	.01 → 1.00	(0.99)
Diluted 1-30	—	0.200	—	—	—	.01 → 0.300	(0.29)
Adj. $\bar{c}$ pH 4.67 1-30	—	—	0.200	—	—	.02 → 0.300	(0.58)
Adj. $\bar{c}$ pH 5.2 1-30	—	—	—	0.200	—	.02 → .600	(0.58)
H <sub>2</sub> O to V <sub>f</sub> = 1.00	0.085	0.085	0.085	0.085	0.285X		
Total cpm / incub =	107,200						
s.a. P	= 17,900						
cpm - blank = 20,0	537	614	<del>645</del> <sup>593</sup>	504			
Total cpm inc	5910	6760	<del>6740</del> <sup>6530</sup>	5540			
$\mu$ moles P <sup>32</sup> inc. / mt.	.330	.378	<del>.377</del> <sup>.365</sup>	.310			
$\mu$ moles P <sup>32</sup> inc. / ml.	165	156.7	<del>156</del> <sup>154.6</sup>	146.4			
Total units	165	159	156	137			

Singer  
Hilmoe  
5-8-58

21  
pg. 20

Large Scale  $\text{Ca}_3(\text{PO}_4)_2$  Gel

4-22-DR<sub>58</sub>, centrifuged. 28 mg/ml. 165 U/ml. (All additions are magnetic stirrer).  
10 ml  $\equiv$  280 mg prot. Dilute to 28 ml. with 0.01 M  $\text{KPO}_4$ , pH 7.4  
Put 0.01 ml. aside for assay.

Add 1M NaAc, pH 5.2 - 1.0 ml - pH = 6.25 (12°)  
0.3 ml 5.99 (11°)  
0.2 ml 5.92 (11°)  
0.2 ml 5.86 (11.5°)  
0.2 ml 5.77 (10.5°) (Remove 0.01 ml. aliquot).

Slightly Turbid. Spin. 15 min, 13,000 rpm. 21,600 x g. (Lourdes).  
small gelatinous precipitate (0.01 ml aliquot of supernatant)

I ADD 2.5 ml.  $\text{Ca}_3(\text{PO}_4)_2$  gel (22.8 mg/ml) over ~ 5 min.  
Stir 20 min. more.

Centrifuge 10 min. 13,000 rpm, 21,600 x g (Lourdes).

Sup I = 24.9 ml. pH = 5.8 (14.5°). Remove 0.02 ml. for assays.

El I. elute  $\bar{c}$  3.75 ml. 0.1 M  $\text{KPO}_4$ , pH 6.0 (5 min. stirring at rm. temp, 20 min. in ice) Centrifuge as above. Repeat with pH 7.4 buffer - 0.1 M, and repeated by exper  $\bar{c}$  0.01 M  $\text{KPO}_4$  pH 7.4 = El I<sub>c</sub> = 3.7 ml - colorless.

II To sup I, add ~~5.2~~<sup>6.5</sup> ml gel. over ~~7.5~~<sup>7</sup> min. Stir 20 min. more.

Centrifuge as above Lourdes 13000 rpm 21600 x g.

Sup II = 35.4 ml. pH = 5.9 (12°). Add 1M Acetate pH 5.2 - 0.2 ml pH = 5.79 (14.5°);  
0.1 ml pH 5.8 (12°).

Gel II, Elute, as above. (0.1 M  $\text{KPO}_4$ , pH 6.0, 3.75 ml.) =  
then with 0.01 M  $\text{KPO}_4$ , pH 7.4 3.75 ml = El II<sub>b</sub> = 3.5 ml - sl yellow.  
then with 0.1 M  $\text{KPO}_4$ , pH 7.4 3.75 ml = El II<sub>c</sub> = 3.6 ml - more yellow.

III To Sup II after adj to pH 5.8, add 6.5 ml gel over 7 min, coat stir 20 min.

Cent. as above.

Sup. III = 40.2 ml - pH = 5.85 (11°) - frozen.

Hel III - Elute as above (total 28 min) (0.1 M  $\text{KPO}_4$ , pH 6.0, 3.75 ml) = El III<sub>a</sub> - 3.8 ml - yellow.  
then with 0.1 M  $\text{KPO}_4$ , pH 7.4, 3.75 ml = El III<sub>b</sub> - 3.5 ml - yellow



# Protein Assay (Phenol) - 5-4-58.

	<u>DILUTION</u>	<u>Sple</u>	<u>H<sub>2</sub>O</u>	<u>d<sub>660</sub></u>	<u>-BL</u>	<u>mg/aliquot</u>	<u>mg/cc.</u>
① pH 5.7 after cent.	1:30	<del>0.065</del> 0.065	<del>435</del> 435	.202	.142	.0222	10.2
② Sup I	1:50	.10 <sup>✓</sup>	.40	.211	.151	.0236	11.8
③ Sup II	1:20	.10 <sup>✓</sup>	.40	.219	.159	.0249	5.0
④ Sup III	1:5	.07 <sup>✓</sup>	.43	.330	.270	.0422	3.0
⑤ El. Ia	1:20	.10 <sup>✓</sup>	.40	.204	.144	.0226	4.51
⑥ El. Ib	1:20	.10 <sup>✓</sup>	.40	.200	.140	.0219	4.38
⑦ El. IIa	1:100	.30 <sup>✓</sup>	.20	.281	.221	.0346	11.5
⑧ El. IIb	1:50	.20 <sup>✓</sup>	.30	.220	.160	.025	6.2
⑨ El. IIc	1:50	.20 <sup>✓</sup>	.30	.250	.190	.0297	7.42
⑩ El. IIIa	1:10	.10 <sup>✓</sup>	.40	.284	.224	.035	3.5
⑪ El. IIIb	1:10	.10 <sup>✓</sup>	.40	.400	.340	.053	5.3
⑫ Std. pl. alb. 2.8mg/ml.	—	0.01 <sup>X</sup>	.49	.239	.179	.028	
⑬ " "	—	0.02 <sup>X</sup>	.48	.402	.342		
⑭ Blank	—	—	.50	.060			
⑮ "	—	—	.50	.060			

Singer  
Helmoe  
5-8-58

Assays on Gel Step

p. 22

(new R-Mix - ~~1/10~~ of 5-5-58)

	1	2	3	4	5	6	7	8		
R-Mix 5-8-58	0.300	—————						→	X	
KH <sub>2</sub> PO <sub>4</sub> (0.01M)	0.340	—————						→	X	
ADP 34 μm / M	0.075	—————						→	X	
Centr 4-22-DR <sub>58</sub>	0.200									.01 → 1.00 (.99)
Diluted 4-22-DR <sub>58</sub>		0.200								.01 → 0.300 (.29)
PH Adj. 4-22-DR <sub>58</sub>			0.200							.01 → 0.300 (.29)
Sup I del 1-50				0.200						.02 → 1.00 (.98)
Sup II del 1-20					0.200					.02 → .400 (.38)
EI Ia 1-20						.200				.02 → .400 (.38)
EI Ib 1-20							.200			.02 → .400 (.38)
EI <sup>H<sub>2</sub>O h<sub>2</sub>O = 100</sup> <del>1-100</del>	.085	—————						→	<del>1.200</del> 1.285	.01 → 1.00 (.99)
cpm - bl. <sup>17.8</sup> μi	492	497	500	150	133	104	46	<del>231</del>		
- Control	471	476	479	129	112	83	19			
Total cpm inc.	5180	5230	5260	1420	1230	912	209			
Total cpm / incub.	= 90,900									
Total P / incub.	= 6.00									
s.a. P <sup>32</sup> =	15,200									
μmoles P <sup>32</sup> incorp.	.341	.345	.346	.093	.081	.060	.014			
μmoles P <sup>32</sup> incorp.	170	51.7	51.9	23.2	8.1	6.0	1.4			
μmoles P <sup>32</sup> / mg enz	6.1	5.2	5.1	1.9	1.6	1.3	.32			
Total <sup>ml. fract.</sup> units	10 ml	28	29.9	29.9	35.4	~3.6	~3.6			
Total prot.	280	280	305	353	177	16.2	15.8			
Total units	1710	1450	1560	670	283	21.1	5.1			



Assays on Gel step (cont). p. 23

Singer  
Helmoe  
5-8-58

(Use sig. data, control of pg. 22)

	9	<del>10</del>	<del>11</del>	<del>12</del>	<del>13</del>	<del>14</del>	<del>15</del>	<del>16</del>	
R-Mix <sup>5-8-58</sup>	0.300								→ X
KH <sub>2</sub> PO <sub>4</sub> (0.01M)	0.340								→ X
ADP 34 μm/ml	0.075								→ X
pH adj. <sup>pH 5.77</sup> 1-30	0.200								.01 → .300 (.290)
Sup III 1-5		0.200							.11 → .5 (.400)
EL II a 1-100			0.200						.01 → 1.00 (.99)
EL II b 1-50				1200					.01 → .500 (.49)
EL II c 1-50					1200				.01 → .500 (.49)
EL III a 1-10						.200			.85 → .50 (.45)
EL III b 1-10							.200		.105 → .50 (.45)
H <sub>2</sub> O to vol 1.00	.095								→ 1285 X
Cpm - blank = 17.8	477	100	339	96	132	850	610		
- Control = 21	456	79	318	75	111	829	589		
Total cpm / incub	5020	970	3500	825	1220	9110	6490		
μM P <sup>32</sup> incorp.	.331	.057	1230	.054	.080	.599	.426		
μM incorp / M enz	49.6	14	115	13.5	20	29.9	213		
μm s.a.	5.2	.5	10	2.2	2.7	8.5	4.0		
Total vol of fract	29.9	40.2	~3.6	3.5	3.6	3.8	3.5		
Total prot	280	120	41	21.7	26.7	13.3	18.5		
Total units	1460	60	410	47.7	72.2	113	74		

	mg prot	units		mg prot	units		mg prot	units
Sup I	353	670	Sup II	177	28.3	Sup III	120	60
EL II a	16	21	EL II a	41	410	III a	13	113
EL II b	16	5	EL II b	22	48	III b	19	74
	385	696	EL II c	27	72		152	247
				267	813			

Do (assay)

II a several levels.  
Sup III several levels.

Assays on Gel Step (cont)

To be sure assay is linear.

cpm/incub. = 84,500  
P<sub>inc</sub> = 6.00 so P<sub>32</sub> = 14,100

	1	2	3	4	5	6	7	8	9	
R-Mix	.300	—————→ X								
KH <sub>2</sub> PO <sub>4</sub> (0.01N)	.300	—————→ X								
ADP 3 μm/ml	.075	—————→						-	-	.075 X
Sup III	1.050	.10	.20	-	-	-	.200	-	-	
EL II a 1-50	-	-	-	<del>.200</del>	<del>.100</del>	<del>.050</del>	-	.200	-	
H <sub>2</sub> O 1.00d	.235 <sup>v</sup>	.185 <sup>v</sup>	.085 <sup>v</sup>	.085	.185 <sup>v</sup>	.235 <sup>v</sup>	.160	.160	.285 <sup>v</sup> X	
Total										
cpm - bkgd = 18.8	122	268	613	779	339	143	56.6	8.8	21.1	
- no enz	101	247	592	758	318	122				
- no sub.	87	<del>170</del>	535	749	309	113				
Total cpm inc.	958	2040	5880	8250	3400	1240				
μmP <sub>32</sub> inc.	.068	<del>.17</del>	.42	.59	.24	.085				
μmP <sub>32</sub> inc / m enz	1.4	<del>1.7</del>	2.1	147	120	88				
su	.154	.65	.81	27	22	16	(proteins: pg 25)			



MON. 12 MAY 1958.

REPEAT PROTEINS (PHENOL) ON GEL FRACTIONS OF 5-8-58.  
NO TRIS BUFFER - DILUTIONS IN H<sub>2</sub>O.

	DILUTION	SPL	H <sub>2</sub> O	660nm net		mg/plate	mg/ml	
				10'	15'			
①	Sup III	1:5	.06✓	.44	.252	.189	.0311	2.6
②	Elvate Ia	1:10	.08✓	.42	.200	.137	.0226	2.8
③	Elvate Ib	1:10	.08✓	.42	.192	.129	.0213	2.7
④	Elvate IIa	1:20	.04✓	.46	.132	.069	.0114	5.70
⑤	" "	1:20	.07✓	.43	.177	.114	.0188	5.37
⑥	Elvate IIb	1:10	.05✓	.45	.108	.045	.0074	1.48
⑦	" "	1:10	.07✓	.43	.127	.064	.0105	1.50
⑧	Elvate IIc	1:10	.04✓	.46	.178	.115	.0190	4.25
⑨	" "	1:10	.06✓	.44	.222	.159	.0262	4.37
⑩	Elvate IIIa	1:5	.06✓	.44	.278	.215	.0354	3.0
⑪	Elvate IIIb	1:10	.08✓	.42	.289	.226	.0372	4.7
⑫	Sld pl. alt. 2.8 mg/ml	—	.01✓	.49	.237	.174	.340 = .056 mg	
⑬	" "	—	.02✓	.48	.394	.331		
⑭	Blank	—	—	.50	.067	.063		
⑮	" "	—	—	.50	.058			

Dialyze elvate IIa, IIb against ~ 5l 0.01 N KHP0<sub>4</sub>, pH 7.4,  
 rocking dialyzer, running buffer. Start 5 PM. Rocker  
 stopped sometime after 10 PM. Dialysis stopped  
 9<sup>15</sup> AM, 5-13-58.

IIa 3.9 ml.  
 IIb 3.5 ml.

Assays on Dialyzed Gel Eluates

	1	2	3	4	5	6	7	8		
R-Mix	0.300	—————→							X	
0.01M $KH_2PO_4$	0.340	—————→							X	
ADP $34 \mu M / l$	0.075	—————→						-	-	X
ELIa, Dial <sup>1-50</sup>	0.200	0.100	0.050	-	-	-	0.200	-	.02 → 1.00	
ELIb Dial <sup>1-10</sup>	-	-	-	0.200	0.100	-	-	0.200	.1 → 1.00	
H <sub>2</sub> O	.085	.185	.235	.085	.185	.285	.160	.160	X	
Total cpm/inc. =	75,600,		P = 6.0		sa P <sup>32</sup> =		12,600			
cpm/inc - Bkgd <sup>Bkgd = 22.7</sup>	473	197	92	417	159	2.0	12.2	1.4		
- no enz	471	195	90	415	157					
- no sub.	459	183	78	414	156					
Total cpm inc.	4940	2010	859	4560	1720					
$\mu M P^{32}$ inc.	<del>1.392</del> 1.392	.159 <del>.253</del>	1.068	1.362	.136					
$\mu M P^{32} / \mu M enz$	98	78	68	18.1	13.6					
sa.	24.5	19.5	17.0	12.1	9.1					

not pg 27.



TUES 13 MAY 1958.

PROTEINS (PHENOL) - DIALYZED GEL PREPS +  
p927 3% - EtOH FRACT, 4-22-DR.

		DILUTION	SPLF	H <sub>2</sub> O <sup>✓</sup>	OD <sub>260</sub> <sup>10</sup>	-A <sub>280</sub>	mg prot	mg/ml	
①	4-22-DR 3% - EtOH cent.	1:100	0.10 <sup>✓</sup>	.40	.240 <del>.232</del>	.168 <del>.172</del>	.028	28	26
②	" "	"	0.20 <sup>✓</sup>	.30	.360 <del>.352</del>	.288	.048	24	
③	ELUATE IIa DIALYZED 5-13-58	1:10	0.06 <sup>✓</sup>	.44	.217 <del>.208</del>	.144	.024	4.0	
④	" "	"	0.10 <sup>✓</sup>	.40	.310 <del>.301</del>	.237	.039	3.9	
⑤	ELUATE IIb DIALYZED 5-13-58	-	0.02 <sup>✓</sup>	.48	.223 <del>.231</del>	.159	.029	1.5	
⑥	" "	-	0.03 <sup>✓</sup>	.47	.307 <del>.308</del>	.244	.041	1.4	
⑦	Std pl. alb. 2.8 mg/ml	-	0.01 <sup>✓</sup>	.49	.240 <del>.232</del>	.168	.028		
⑧	" "	-	0.02 <sup>✓</sup>	.48	.391 <del>.383</del>	.319	.056		
⑨	Blank	-	-	.50	.061 <del>.060</del>				
⑩	"	-	-	.50	.074 <del>.068</del>				

GEL

10 ml. 4-22-DR<sub>58</sub> centr. (25 mg/ml)  
 10 ml = 250 mg prot. dilute to 28 ml in 0.01 M KPO<sub>4</sub>, pH 7.1  
 containing 0.0015 M cysteine.

ADD 1 M NaAc, pH 5.2 - 1.4 ml - pH = 5.97 (13°)  
 TOTAL → 1.8 ml - pH = 5.70 (10°)

dil. 0.1 → .50  $\frac{Abs_{280}}{Abs_{260}} = \frac{.588}{.815} = .72 \equiv .299 \text{ mg/ml} \equiv 14.9 \text{ mg prot/ml}$

I ADD 1.25 ml Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> gel (22.8 mg/ml) over 5 min, stir (mag.) 20 min total.  
 Cent 10 min 13000 rpm, 21,600 x g - LOURDES.  
 Sup I = 29.4 ml - pH = 5.74 (14°) Remove 0.05 ml (5) splcs.

$\frac{280}{260} \cdot \frac{.527}{.735} = .72 \equiv .268 \text{ mg/ml} \equiv 13.4 \text{ mg prot/ml}$

Gel I - Elute @ 3.75 ml. 0.1 M ~~NaAc~~ KPO<sub>4</sub>, pH 7.4. 20 min. 3.6 ml. ELUATE I<sub>a</sub>  
 Centrifuge 10 min, 13,000 rpm, 21,600 x g (Lourdes).

El I, dilute .03 → 1.00  $\frac{280}{260} = \frac{.213}{.287} = .74 \equiv .108 \text{ mg/ml} \equiv 3.6 \text{ mg/ml}$

II To Sup I, add 1.25 ml gel as above - over 5 min, cont. stir 20 min.  
 Centrifuge as above - Serrall - 10 min - pos 120.

Sup II = 30.3 ml - pH = 5.79 (12°), Remove 0.02, 0.02, 0.01 ml splcs.

dilute 0.1 → .50  $\frac{280}{260} = \frac{.488}{.685} = .71 \equiv .234 \text{ mg/ml} \equiv 11.7 \text{ mg prot/ml}$

Gel II Elute @ 3.75 ml 0.1 M KPO<sub>4</sub> pH 6.0 20 min. (as above) EL II<sub>a</sub><sup>3.6 ml</sup>  
 " " " " " " " " " " EL II<sub>b</sub><sup>3.6 ml</sup>  
 " " " " " " " " " " pH 7.4

EL II<sub>a</sub>  $\frac{280}{260} = \frac{.1592}{.1925} = .64 = .223 \text{ mg/ml} \equiv 2.23 \text{ mg/ml}$

III To Sup II, add 7.5 ml gel as above - over 5 1/4 min, cont stir 20 min.  
 → Sup III = 36.0 ml - pH = 5.9 (11°) ADD 1 M NaAc pH 5.2 - 0.2 ml - pH = 5.96 (11°) + 0.2 ml - pH = 5.72 (11°)

105 → 50  $\frac{280}{260} = \frac{1.415}{1.94} = .73 \equiv .72 \text{ mg prot/ml} \equiv 7.2 \text{ mg/d}$

GEL III Elute @ ~~3.75~~<sup>5.0</sup> ml 0.1 M KPO<sub>4</sub> pH 6.0 - ca 30 min (as above) EL III<sub>a</sub> = 5.0 ml  
 " " 5.0 ml 0.1 M " pH 7.4 - 25 min total (as above) EL III<sub>b</sub> = 5.0 ml  
 " " 5.0 ml 0.1 M KPO<sub>4</sub> pH 7.4 - 6 + 20' (as above) EL III<sub>c</sub> = 4.8 ml.

EL III<sub>a</sub> + III<sub>b</sub> = yellow; EL III<sub>c</sub> - nearly colorless.



IV - 9/6 Sup III, after adj pH - add 6.5 ml gel as above - over 5 min  
 cont. stirring for 25 min. Cent 13000 rpm 21, 600 x g. 1 hour 08.  
 Sup IV = 41.0 ml - pH = 5.88 (110)

GEL IV - Elute @ 5.0 ml 0.1M KPO<sub>4</sub> pH 7.4 - 5' + 35' (as above) El IV a = 5.0 ml.

$\frac{d_{280}}{d_{260}}$	DILUTION	$d_{280}$	$d_{260}$	$\frac{d_{280}}{d_{260}}$	$\frac{1.5 d_{280} - .75 d_{260}}{1.57}$	approx total prot/ml.
El. II b	.05 <sup>v</sup> → .5 <sup>v</sup>	.278	.347	.802	.417 - .260 = .157	1.57
El. III a	.05 <sup>v</sup> → .5 <sup>v</sup>	.990	1.397	.708	1.485 - 1.047 = .438	4.38
El. III b	.05 <sup>v</sup> → .5 <sup>v</sup>	1.858	2.72(?)	.68(?)	2.78 - 2.040 = .740	7.40
El. III c	.05 <sup>v</sup> → .5 <sup>v</sup>	.332	.533	.623	.498 - .399 = .099	0.99
Sup IV	.02 <sup>v</sup> → .5 <sup>v</sup>	.422	.600	.704	.632 - .450 = .182	4.55
El. IV a	.05 <sup>v</sup> → .5 <sup>v</sup>	.906	1.134	.799	1.360 - .850 = .510	5.10
El. III a	.02 <sup>v</sup> → .5 <sup>v</sup>	.400	.559	.716	.600 - .419 = .181	4.53
El. III b	.01 <sup>v</sup> → .5 <sup>v</sup>	.400	.658	.608	.600 - .494 = .106	5.30
El. IV a	.025 <sup>v</sup> → .5 <sup>v</sup>	.448	.553	.810	.672 - .414 = .258	5.16

Eluate III a - ca 4.8 ml next @ NH<sub>4</sub>OH -

0.1 N NH<sub>4</sub>OH - 0.03 ml

0.05 ml

1 N NH<sub>4</sub>OH - 0.01 ml

0.01 ml

0.02 ml

0.02 ml

1.15 ml

yellow green BTB

" " "

" " "

(Sple in orig tube for protein)

Assays - Gel Step

New R-MIX 3 ml 1 M Tris, 8.2, 1.5 ml 0.1 M MgCl<sub>2</sub>, 0.6 ml 0.05 M Versene, P 32, 0.10 ml Chyd 5-14-58, no carrier) + 3.80 ml H<sub>2</sub>O.

	1	2	3	4	5	6	7	8	Total cpm/incub =	
R-mix <sup>5-16-58</sup> <del>5-8-58</del>	.300	—————							X	86,400
0.01 M <sup>2</sup> CaPO <sub>4</sub>	.350	—————							X	P = 6.0
ADP <sup>34</sup> <del>μmole</del>	.075	—————							X	s.d. = 14,400
Acer pH <sup>1-30</sup>	.200								101 → .300 (1.29)	
Sup I <sup>1-25</sup>	<del>.200</del>	.200							.02 → .50 (1.48)	
ER I <sup>1-10</sup>			.200						.05 → .50 (1.45)	
Sup II <sup>1-25</sup>				.200					.02 → .50 (1.48)	
ER IIa <sup>1-10</sup>					.200				.05 → .50 (1.45)	
ER IIb <sup>1-10</sup>						.200			.05 → .50 (1.45)	
Sup III <sup>1-10</sup> <del>H<sub>2</sub>O</del>							.200		.03 → .30 (1.27)	
H <sub>2</sub> O <sup>1.00M</sup>	.075	.075	.075	.075	.075	.075	.075	.275	<del>.285</del> X	
cpm - blank = 22	493	525	43	374	40	30	111	5.9		
- Control	487	519	37	368	34	<del>33</del> <sup>24</sup>	105			
Total cpm incorp.	5350	5710	406	4050	374	264	1150			
μmPinc.	.372	.396	.028	.281	.026	.018	.080			
μmPinc./ml enz	55.8	49.5	1.4	35.1	1.3	.9	4			
Total vol.	29.8	29.4	3.6	30.3	3.6	3.6	36.0			
Total units	1660	1450	<del>1005</del>	1060	5	3	144			
s.d.	7.2	6.9	.5	5.4	1.7	.6	1.0			
Total prot.	232	212	10.5	197	6.8	5.0	144			



# ASSAYS - GEL STEP.

SINGER  
HILMOZ  
5-16-58

page 30

	9	10	11	12	13	14
R-mix 5/16-58	0.300	—————				X
0.01M $K_2HPO_4$	0.340	—————				X
ADP 34M/ml	0.075	—————				X
El III <sub>a</sub> 1-100	0.200					
El III <sub>b</sub> 1-10		0.200				
El III <sub>c</sub> 1-10			0.200			
Sup IV undil				0.200		
El IV <sub>a</sub> 1-20					0.200	
H <sub>2</sub> O to 1/4 100	.085	—————				X, 285
cpm - bkgd = 222	314	696	135	601	203	
- Control	308	690	129	595	197	
Total cpm enc.	3390	7600	1420	6550	2170	
$\mu$ MPE enc	.236	.527	.099	.455	.150	
$\mu$ MPE/dec.	118	264	5.0	228	15	
Total vol	5	5	4.8	41	5	
Total units	590	132	24	93.5	75	
S. a.	30.2	5.0	5.0	.85	3.4	
Total prot.	19.5	27	5.0	111	22.0	

S. a.  $P^{32} = 14,400$

.01 → 1.00 (.99)  
 .05 → .50 (.45)  
 .05 → .50 (.45)  
 —  
 .05 → 1.00 (.95)

5-19-58

PHENOL PROTEINS (from 5-16-58)

	DILUTION	SPLF	H <sub>2</sub> O	660 nm	-B	mg prot	(diluted) mg prot/ml	units mg/ml
.02+ 58 ① After pH adj. -	1:30	0.2ml	.3	398.380	.341	.052	.26	27.8
.02+ 58 ② Sup I	1:30	0.2ml	.3	371.360	.321	.049	.24	7.2
.02+ 48 ③ Sup II	1:25	0.2ml	.3	388.372	.333	.051	.26	6.5
.1+g ④ Sup III	1:10	0.1ml	.4	310.299	.260	.040	.40	4.0
⑤ Sup IV	1:10	0.1ml	.4	220.212	.173	.027	.27	2.7
⑥ Elution Ia undil		0.01ml	.49	233.227	.188	.029		2.9
⑦ " IIa		0.02ml	.48	299.291	.252	.039		1.9
⑧ " IIb		0.02ml	.48	228.221	.182	.028		1.4
⑨ " IIIa		0.01ml	.49	300.291	.252	.039		3.9
⑩ " IIIb	1:10	0.07ml	.43	289.277	.238	.037	.53	5.3
⑪ " IIIc undil		0.02ml	.48	177.170	.131	.020		1.0
⑫ " IVa		0.01ml	.49	338.328	.289	.044		4.4
⑬ Std ph. alb. 2.8mg/ml -		0.01ml	.49	276.221	.182			
⑭ " " " "		0.02ml	.48	412.402	.363			
⑮ Blank		-	.50	99.039				
⑯ "		-	.50	99.039				

Resume

Gel, 5-16-58

Start: 1660 units, 232 mg prot.

	units	mg
Sup I	1450	212
El I	5	10.5
	<u>1455</u>	<u>222.5</u>
Sup II	1060	197
El II a	5	6.8
II b	3	5.0
	<u>1068</u>	<u>208.8</u>

	units	mg
Sup II	144	144
El III a	590	14.5
III b	132	27
III c	24	5.5
	<u>890</u>	<u>196.0</u>
Sup IV	94	111
El IV a	75	22
	<u>169</u>	<u>133</u>

5-19-58

Dialyze III a vs. 0.01 M KPO<sub>4</sub>, pH 7.4  
 5<sup>20</sup> pm - 9<sup>30</sup> AM (5-20-58). 4.8 ml at start.  
 7.0 ml at finish. (running buffer, 400-500 ml/hr)  
 Used ca 7 liters 0.01M KPO<sub>4</sub> pH 7.4



Final Gel Fractionation on Remaining Zn-ETOH II  
Procedure as of 5-16-58, with change as noted.

4-22-DR<sub>58</sub>, centrifuged. 17.5 ml.

Dilute to 49 ml. with 31.5 M. 0.01 M KPO<sub>4</sub>, pH 7.4, containing 13.2 mg. cysteine, HCl. H<sub>2</sub>O (0.0015 M in cysteine), final pH about 7.1.

Add 1 M NaAc pH 5.2 (40) - <sup>2.62</sup> 1.5 ml - pH = 6.32 (13.5)  
0.5 ml - 5.98 (120°)  
0.32 5.88 (110)  
0.40 5.80 (110)  
0.30 5.70 (100)

538  
dil .01 → .50  $\frac{280}{260} = \frac{1582}{1809} = 172 \equiv .296 \text{ mg prot/ml} \equiv 14.8 \text{ mg prot/ml.}$

I. Add 2.19 ml Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> gel (22.8 mg/ml) - over 5 min, cont. stir 20 min - mag. stir in ice - Cent 13000 rpm, 21,600 xg - Lourdes 10'

Sup I pH 5.78 (130) = 52.8 ml.

dil .01 → .50  $\frac{280}{260} = \frac{1565}{1790} = 172 \equiv .287 \text{ mg prot/ml} \equiv 14.3 \text{ mg prot/ml}$

II. To Sup I add 2.19 ml Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> gel, as above.

Sup II = 53.8 ml - pH 5.77 (110)

101 → .50  $\frac{280}{260} = \frac{1496}{1704} = 171 = .237 \text{ mg/ml} \equiv 11.9 \text{ mg/ml.}$

III \$0 Sup II Add 13.1 ml gel, as above.

Sup III = 64 ml.

.02 → .50  $\frac{280}{260} = \frac{1571}{1798} = 172 = .299 \text{ mg/ml} \equiv 7.5 \text{ mg/ml.}$

GEL III - Elute = 8.75 ml 0.1 M KPO<sub>4</sub> pH 6.0 - 10' to susp - 20' to clear stir = EL. III<sub>a</sub> = 8.8 ml.

.02 → .500  $\frac{280}{260} = \frac{1386}{1545} = 171 \equiv .185 \text{ mg/ml} \equiv 4.62 \text{ mg/ml}$

Elute = 8.75 ml 0.01 M KPO<sub>4</sub> pH 7.4 - 6' to susp, 20' to clear stir = EL. III<sub>b</sub> = 8.8 ml

~~8.75 ml 0.1 M KPO<sub>4</sub> pH 7.1~~

(OVER)

Dialysis vs. 0.01M KPO<sub>4</sub> pH 7.4. - Start 5<sup>00</sup> pm (9.2 ml/min).  
 Stop 9<sup>30</sup> AM (5-22-58). - Had used 8 liters 0.01M KPO<sub>4</sub> pH 7.4.

Slow rate = 5-21 @ 5 pm = 9.2 ml/min; 5-22 @ 9<sup>30</sup> am = 6.5 ml/min.

2K - green marble = Eluate III<sub>a</sub> - ca 8.5-8.7 ml. → 10.4

3K - blue marble = Eluate III<sub>b</sub> - ca 8.5-8.7 ml → 11 ml.

} 5-22  
58

5-22-58

Phenol Proteins

	Dil	sple	H <sub>2</sub> O	<sup>0.10</sup> <del>0.10</del> 0.10	-BI	mg/sple	mg/ml
✓ ① After pH adj.	1-30	0.15	0.35	.312	.265	.0405	8.1
✓ ② EL III <sub>a</sub> (5-21-58) undil		0.01	0.49	<sup>0.02</sup> .294	.247	.0378	3.8
✓ ③ EL III <sub>b</sub> (5-21-58) undil		0.01	0.49	<sup>0.148</sup> <del>.144</del> 0.144	.1097	.0148	1.5
✓ ④ EL III <sub>b</sub> (5-21-58) undil		0.02	0.48	<sup>0.36</sup> .231	.184	.0282	1.4
✓ ⑤ EL III <sub>a</sub> (5-21-58) undil Dialyzed		0.015	0.485	<sup>0.360</sup> .357	.310	.0474	3.2
✓ ⑥ EL III <sub>b</sub> (5-21-58) undil Dialyzed		0.02	0.48	<sup>0.215</sup> .208	.161	.0246	1.2
✓ ⑦ EL III <sub>a</sub> (5-16-58) undil Dialyzed		0.015	0.485	<sup>0.322</sup> .319	.272	.0416	2.9
✓ ⑧ Std, Plasma Alb 2.8 mg/ml		0.010	0.49	<sup>0.232</sup> .230	.183		
⑨ BL		-	0.500	<sup>0.044</sup> .047			



Assays on  $Ca_3(PO_4)_2$  Gel Step.

Block = 21.8  
S.A. P<sub>32</sub>, 11,200

15 min. 37°C.

	1	2	3	4	5	6	7	8		
R-Mix, 5-16-58	.300	_____							X	
0.01M $K_2HPO_4$	.350	_____							X	
ADP 34 $\mu$ m/ml	1.075	_____							X	
After pH	1-30								-	
Sup I	1-25	.200						-		.01 → .30 (.29)
Sup II	1-15		.200					-		.02 → .50 (.48) (.28) .02 → .30
Sup III	1-5			.200				-		.11 → .15 (.14)
EL III a	1-100				.200			-		.01 → 1.00 (.99)
EL III b	1-10					.200		-		.03 → .300 (.27)
EL III c	1-10						.200	-		.03 → .300 (.27)
$H_2O$ to V <sub>f</sub> = 100 <sup>m</sup>	1.075	.075	.075	.075	.075	.075	.075	.275	X	
cpm - block	414	447	535	214	306	460	<del>24</del>	29		
- control	385	418	506	185	277	431				
Total cpm incorp	4240	4610	5560	2030	3070	4740				
$\mu$ M P incorp	.378	.412	.496	.181	.274	.424				
$\mu$ M P inc/ml enz	56.6	51.6	37.3	4.5	137	212				
Total vol.	52	52.8 <del>51.2</del>	53.8	64	8.8	8.8				
Total units	2940	2730 <del>2660</del>	2010	288	1210	187				
Total prot	421				33.4	12.3				
S.A.	7.0				36.2	15.2				

THURS. 22 MAY 1958.

Solutions for Protamine Step on Cy. Poly. phosphorose.

20%  $(NH_4)_2SO_4$   $\approx$  1% glycine pH 6.2  
(M.W. = 75.07)

40 ml saturated A.S. + 1.5 gm glycine +  
~~125~~ 125 ml  $H_2O$  - pH = 5.77 (17°)  
add 0.2 N  $NH_4OH$  - 1.0 ml - pH = 6.87 (15°)

add 0.1 N HAc

0.4 ml	pH = 6.82 (14°)
1.0 ml	6.33 (12°)
0.2 ml	6.16 (10.5°)
<u>1.6 ml</u>	

diluted to 200 ml - pH = 6.05 (17°)  
6.19 (13°)  
6.18 (10°)

2% Protamine pH 5.9

0.4 gm Protamine- $SO_4$  Lilly Lot #6069 -  
552227 suspended in 15 ml  $H_2O$  -  
pH  $\approx$  7.4 (melted)

add 0.1 N HAc -

0.05 ml	pH = 6.6 (24.5°)
0.03 ml	6.32
0.04 ml	5.90
<u>0.12 ml</u>	

dilute to 20 ml - pH = 5.98  
add 0.01 ml 0.1 N HAc pH = 5.87



Singer  
5-23-58

pg 33

Assays on Dialyzed Gel Fractions

SA  $P^{32}$  9980 cpm/ $\mu$ mole.

	1	2	3	4	5	6	7
R-Mix 5-16-58	.300	.300	.300	.300	.300	.300	.300 X
0.01M $K_2HPO_4$	.350	.350	.350	.350	.350	.350	.350 X
ADP 34 $\mu$ m/ml	.075	.075	.075	.075	.075	.075	<del>.075</del> X
IIa, 5-21-58 Dial <small>0.1 1-70</small>	-	.200	.100	-	-	-	- X
IIIb 5-21-58 Dial <small>0.1 1-10</small>	-	-	-	.200	-	-	- X
IIIa 5-16-58 Dial <small>0.1 1-70</small>	-	-	-	-	.200	.100	.200
H <sub>2</sub> O	.275	.075	.075	.075	.075	.175	.150 X
cpm - Blank = 26.7	37.8	222	129	285	196	116	29.3
- Control = 38		184	91	247	158	78	
Total cpm		2025	1000	2720	1740	858	
$\mu$ M P incorp.		.203	.160	.273	.175	.086	
$\mu$ M P incorp/menz.		71	70	13.7	61	60	
$\mu$ M P incorp/mgenz		22.2	21.9	11.4	21.8	21.4	

Perhaps should dialyze vs. cysteine - phosphate?

Singer  
Hirsch  
5-26-58

Protamine etc

Combine 5-21-58	III <sub>a</sub> EL (dial)	3.2 mg/ml	10 ml.	32 mg
5-16-58	III <sub>a</sub> EL (dial)	2.8 mg/ml	6.6 ml	19 mg.
5-13-58	IV <sub>a</sub> EL (dial)	4.0 mg/ml	3.8 ml	15 mg
	(yellow)	3.2 mg/ml		<u>66 mg</u> / 20.4 ml.

In 50 ml. Erlenmeyer

- 1) Add 0.01N HAc (cold, with swirling), 1.5 ml → pH 6.4 (14°).  
     1.75 ml.                      6.01 (12°)  
     1.80 ml                      5.80 (12°).  
     2.05 ml                      5.45
- 2) Add 2% protamine sulfate, adjusted to pH 5.9 ± HAc. - 2.35 ml.  
 Stir in ice, 10 min.
- 3) Centrifuge 10 min. Servall 60/130, (cold room).  
 Supernatant 23.7 ml. (faint yellow)
- 4) Mustard color ppt - (brownish). Mash with small amount of water to suspend. Then total of 2.5 ml H<sub>2</sub>O.  
 Centrifuge as above.  
 2.3 ml H<sub>2</sub>O (colorless)
- 5) Mustard color precipitate. Add 3.5 ml 20% AS containing M/10 glycine, pH 6.2 - (6.18, 10°C). [Final pH 6.18, adj. ± HAc]  
 Most dissolves - yellow colored liquid. (11<sup>45</sup> AM)
- 6) Centrifuge (10<sup>00</sup> AM), servall 50/130, 10 min.  
 3.4 ml yellow, sl. opalescent supernatant. Very small light ppt
- 7) Add 2.04 ml sat. AS soluti. - in 4.5 min. Stand & stirring 18 min.  
 (added 1.5 ml + then 0.005 ml n-amyl alc., then remain 0.54 ml satd A.S. added)
- 8) Cent Servall top speed 15 min. Sup - sl. yellow; ppt - brownish.
- 9) Wash ppt ± 1.55 ml 60% satd A.S., Cent Servall top spd 10 min.  
 Wash - 1.5 ml.

Ppt, taken up in 1.4 ml M/100 phosphate pH 7.4. Get quite a clear slightly opalescent solution. Dilute 100 μM → 100  $\frac{260}{260} = \frac{1.32}{1.17} = 1.13 = .12 \text{ mg/ml} \approx 24 \text{ mg/ml}$   
 Remove 0.02 ml aliquot.



10. To above 1.4 ml add 0.21  $\text{Ca}_3(\text{PO}_4)_2$  gel, (22.8 mg/ml) Stir, 10 min. Centrifuge 10 min. Servall. (10 min, top speed).

11. To yellow sup, add 0.56 ml Alumina CR (Sept 1950, 9.9 mg solids/ml). Stir 10 min. Centrifuge as above. 2.0 ml, clear yellow solution - white gel. pH ~ 6.4 (from ceric purple) (Remove 0.02 ml aliquot)

Dialyze vs running 0.01M  $\text{KH}_2\text{PO}_4$  pH 7.4 with 0.875 gm cysteine. HCl.  $\text{H}_2\text{O}$  per 15 liters (0.0003M) Start 5 PM. [Undialy, dilute 0.01 ml  $\rightarrow$  0.50 (H<sub>2</sub>O)]  $\frac{212}{260} = \frac{.212}{.145} = 1.43 \equiv .212 \text{ mg prot/ml}$   $\frac{0.0003 \text{ M}}{10.6 \text{ mg/l}}$

5-27-58

Stop dialysis 9<sup>15</sup> am. 2.00 ml. [5-27-AR] clear solution - yellow.

Dilute 0.01 ml  $\rightarrow$  0.50 (H<sub>2</sub>O)  $\frac{0.212}{0.138} = 1.54 \equiv .223 \text{ mg prot/ml} \equiv \underline{11.1 \text{ mg/ml}}$

	ml	H <sub>2</sub> O	0.0	-A <sub>2</sub>	mg/spk	mg/d
Phenol Proteins			0.50			
Blank	-	0.500	.048			
Stand (Plasm Alb) 2.8 mg/ml	0.010 ✓	0.490	.228	.180		
	0.020 ✓	0.480	.382	.334		
Final Dialyzed (5-27-AR)	0.015 ✓	0.485	.252	.204	.032	10.5
del 1-5	0.025 ✓	0.475	.365	.317	.053	10.5
Undialyzed	0.015 ✓	0.485	.250	.202	.031	10.5 > 10.8
del 1-5	0.025 ✓	0.475	.368	.320	.054	11.0
41 SP, del 1-10	0.010 ✓	0.490	.178	.130	.020	20 > 19.5
	0.020 ✓	0.480	.270	.242	.038	19

Total protein in 5-27-AR = 21 mg.

Total protein in 41SP = 27.3 mg

ASSAYS ON PROTAMINE - AS (etc)

New R. mix 3 ml 1M Tris, pH 8.2 1.5 ml 0.1M MgCl<sub>2</sub> 0.6 ml 0.05M Versene  
P32, 0.20 ml [hyd 5-14-58, no carrier] + 3.70 ml H<sub>2</sub>O

Blgd = 24.9  
Total cpm = 101,000  
sa p32 = 16,700

	1	2	3	4	5	6	7		
R-Mix 5-27-58	0.300	—————→						X	
0.01M KH <sub>2</sub> PO <sub>4</sub>	0.350	—————→						X	
ADP 34 μm/l Pooled, Dialy Gel eluates. dl 1-70	0.075	—————→						X	
41 SP dl 1-100	0.200						-	(.005 → .35)	
Final, undialyzed (Dialyzed) 527-AR 1-100		0.100					-	(.005 → .5)	
H <sub>2</sub> O to V <sub>f</sub> = 1.00 ml			0.050				-	(.005 → .5)	
H <sub>2</sub> O to V <sub>f</sub> = 1.00 ml	0.075	0.175	0.225	0.225	0.255	0.175	0.275	X	
cpm - blgd.	393	1171	458	207	88	365	114		
- Control	382	1160	447	196	77	354			
Total cpm	4200	12700	4920	2160	848	3900			
μmP encorp.	.251	.765	.295	.129	.051	.234			
μmP enc/ulenz	978	765	590	258	255	234			
sa	27.4	39.2	54.7	24.6	24.3	22.2			
Total vol.	20.4	1.4	2.0	2.0	2.0	2.0			
Protein - mg/ml	6.6	19.5	10.8	10.5	10.5	10.5			
Prot Total	66	27.3	21.6	21.0	21.0	21.0			
Units - Total	1800	1070	1180	516	510	466			

SPECIAL  
GO-OP BOND



Sup from step 8 (5-26-58) which had <sup>5-27-58</sup> been stored frozen -  
 (sup of 50% AS) 5.0 ml.

Add 0.2 ml sat'd AS(0°). Between 1.6 and 2 most of  
 turbidity appears - (not very turbid).

Add 0.12 ml more - more turbidity.

Stand 15 min. cold. Spin 10 min. Servall - top speed  
 Take up ppt in 0.2 ml 0.01M PO<sub>4</sub>, 7.4. Slightly yellow sup.

(also see below).  $\frac{0.250}{0.200} = \frac{583}{1631} =$  5-27 BR, r,   
 5-27 CR, r

	1	2	3
R. MW	300	300	300
0.01M KPO <sub>4</sub>	350	350	350
ADP 34 mg/ml	1075	1075	1075
5-27 BR, r	<del>1000</del>		
5-27 CR, r		.050 <del>1200</del>	.050 <del>1200</del>
Original sup (5-26)			1225 <del>675</del>
H <sub>2</sub> O	1270	1225	1225
ipm-blqd	2095	334	1255
-control	2084	323	1274
Total ipm	22950	3550	14,000
µm Pinc	1.37	.212	.837
µm Pinc/total	274	423	16.8
Total vol	0.2	5.0	5.0
tot units	54.7	215	84.0

4.9 ml 5-27-CR, r + 1.0 ml sat'd  
 A.S. Stand 10' Cent Servall  
 10' top spd. - Sup - mostly  
 colorless, very d. yellow.  
 Ppt taken up in 0.3 ml  
 0.01M KPO<sub>4</sub> pH 7.4 -  
 did not dissolve = suspension  
 = 5-27-DR, r.  
 Supernatant = 5-27-ER, r.

5-27-BR - 0.01 ml → 0.5 ml (turbid)  
 when add 0.5 ml more H<sub>2</sub>O -  
 (still turbid)  $\frac{0.250}{0.200} = \frac{338}{1256} =$

Singer  
Mamks  
5-5-58.

Assays on Protamune: Gel Steps  
pg 36.

	1	2	3	4	5	6	Dilute to 0.05M
R-Mix (5-2758)	0.300	—————→					IMS buffer, pH 8.
0.01 M $K_2H_2PO_4$	0.350	—————→					
ADP 34 $\mu$ M/M	0.075	—————→					15 mM, 37°C
El III b, dialy. 5-21 dil 1-10	0.100	—	—	—	—	—	Ice, adding 0.1M
5-27 BR <sub>58</sub> dil 1-100	—	0.050	<del>0.100</del>	—	—	—	40% Tuckman.
5-27 DR <sub>58</sub>	—	—	—	0.005	0.020	—	
H <sub>2</sub> O to VC = 1.00	0.175	0.225	0.175	0.270	0.255	0.275	✓
- Background cpm (20)	121	191	298	354	1140	20	S.A. P <sup>32</sup> 11,495
- Control	101	171	278	334	1120	—	
Cpm inc. into Nucleotide							
<del><math>\mu</math> mol Pi</del>	<del>0.0088</del>	<del>0.0149</del>	<del>0.0242</del>	<del>0.0384</del>	<del>0.1273</del>	—	
Total cpm	1111	1881	2958	3674	12320	Total cpm	
$\mu$ moles Pi	0.097	0.157	0.258	0.320	1.071		
$\mu$ moles Pi/mg.	<del>8.1</del>	31.4	258	65	53.5		
		$\mu$ moles Pi/mg enzym. sol.					