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ANNUAL PROGRESS REPORT
TO THE UNITED STATES ATOMIC ENERGY COMMISSION

Contract AT(11-1)-3271

MASTER

From the
Department of Biochemistry
The Johns Hopkins University
School of Hygiene and Public Health
Baltimore, Maryland 21205

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Principal Investigator: Roger M. Herriott, Ph.D.
Title: Genetic Transformation in Hemophilus influenzae

This report covers work done during the contractual period
September 15, 1971 to September 14, 1972.

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RESEARCH PROGRAM OF ROGER M. HERRIOTT

I. PURIFICATION OF THE PHOTOREACTIVATING ENZYME FROM YEAST

1. The Starting Material

In an attempt to reduce the effort needed to purify the photoreactivating enzyme from baker's yeast several samples of commercially available dried yeast were extracted in the usual manner and the enzyme concentration compared with our laboratory dried preparation. The Fleischman's commercial dried yeast yielded less than 10 percent of the enzymic activity obtained from fresh Standard Brands yeast that we dried. Red Star dried yeast yielded nearly 30 percent as much as the laboratory dried preps. Should there be a need for a massive quantity of enzyme so commercial dried yeast is needed the Red Star product is the better of the two.

2. Early September Preparations

For two years in a row the enzyme content of extracts of yeast dried in late August and early September seemed to be several fold higher than those obtained earlier or later. We shall test this carefully during the late summer of this year.

3. Improved Procedure

Many small changes in the procedure described last year have been made which do not warrant detailing but they have lent greater reproductibility to the method. The most important one

was the elimination of the preliminary precipitation by streptomycin in the presence of unirradiated DNA. We were losing too much of our enzyme in this step. As seen by the results in Table I the one precipitation by streptomycin of the uv'd DNA - enzyme complex followed by washing the precipitate and dissolving the active principle in 0.25M phosphate buffer yielded a 300-500 fold purification without much loss in activity.

4. Further Purification

A. Solutions #4 in Table I were for a long time difficult to purify further due perhaps to the presence of both RNA and DNA. DNAase seemed to be inhibited by the RNA so the latter was digested by exposure to RNAase after which the photoenzyme was precipitated by the addition of Am SO_4 to bring the solution to 0.8 saturation.

On centrifugation and dissolving the residue and treating it with 0.1 $\mu\text{g/ml}$ DNAase and 5 mM Mg^{++} at room temperature for an hour, then dialysis overnight, we found a precipitate which was inactive and the supernatant was low in nucleic acid. The specific activity (ratio of enzyme activity to protein EU/Pr) increased a little as did the EU/Fl. However this procedure was abandoned in the light of our success described below with the affinity column.

B. Affinity Chromatography

Dialysis of solution #4 for chromatography on uv'd DNA - cellulose according to Minato & Werbin (1971) against A-C buffer yielded a precipitate which proved to be inert protein and was

PURIFICATION OF YPRE⁺

1/ml

Treatment	#	Vol (ml)	EU x10 ³	Pr** mg	Fl***	TEU x10 ⁶	EU/Pr x10 ³	EU/Fl x10 ⁴	Fl/Pr
1500gms air dried baker's yeast extracted with 6 liters 0.05 MK ₂ H PO ₄ + 300ml toluene soluble extract	1	6000	1.2	55		7.2	0.022		
#1 + Am SO ₄ to 0.35 sat + Filteraids; Filtrate and wash solution	2	8000	1.2	35		10.	0.035		
	2a	7800	0.6	18		5	0.035		
Fil #3 + Am SO ₄ to 0.55 sat; Filter; res. diss in 0.05M pH 7.2 PO ₄ ; Dial. against 0.05M PO ₄ =	3	815	8.0	68		6.5	0.12		
another lot	3a	850	3.6	67		3.0	0.05		
#3 + uv'd DNA + streptomycin; ppt. cent, wash and stir with 0.25M pH 7.2 PO ₄ =, cent., super	4	47	140.	5.5	2	6.0	30.0	3	0.36
	4a	50	6.	1.6	0.2	0.3	4.0	3	0.12
Strep. ppt in 4 extracted with 0.1 N PO ₄	4b	10	24.	0.55	0.36	0.24	45.0	7	0.65
4a ppt extracted with 0.25M PO ₄	4c		70.	5.3		3.0	14.0	1.4	
Another lot treated as in #4									
Sample of #4 + Mg ⁺⁺ and DNAase (0.1 µg/ml), dial; add Am SO ₄ to 0.8 sat. cent. Dissolve	5		88.	1.4	0.8		60.	11	0.57
Repeat on another lot.	6		140.	3.2	0.9		44.	15	0.3
Sample of #4 dial against A-C buffer than photo flashed and chromatographed on uv'd DNA cellulose. Elute with 0.5M KCl	7	14.0	2.9	0.005	0.11		600.	2.6	22.
Repe	7a	2.4	9.3	0.004	0.10		2400.	9.0	25.
Next day			3.0				800.	3.1	

Footnotes:

- +
YPRE = yeast photoreactivating Enzyme
- * EU = Enzyme units = arbitrary units obtained from the initial rate of repair of 1 $\mu\text{g/ml}$ 10% active uv inactivated strep. resistance marker. Slope obtained from four points taken at different times of illumination. 1 EU raises the transforming activity of the substrate at the rate of 1×10^6 str. transformant per min. of illumination at 37°C.
- ** Protein by the Lowry Color reaction on 10% TCA precipitate redissolved in 0.5M NaOH.
- *** Fluorescence at 470m μ from 360 m μ excitation in arbitrary units. 1 unit is equal to the fluorescence of 2×10^{-7} Molar Quinine sulfate in 0.1N sulfuric acid.

removed by centrifugation. Upon exposure of the enzymically active supernatant fluid to several photo flashes at close range to release the enzyme from the uv'd DNA in the preparation, the enzyme adsorbed to the affinity column and let the protein impurities wash through. Elution by 0.5M KCl brought the enzyme out with a substantial (10 fold or more) increase in specific activity (EU/Pr). Unfortunately the protein values are so low that their limit of reliability is not better than a factor of 2.

II. FLUORESCENCE OF THE ENZYME

The evidence for fluorescence in the enzyme as reported by Minato & Werbin ('71) add an important dimension to the assay for if the fluorescent component is necessary for enzyme action then the ratio of enzyme activity to fluorescence (EU/F1) might reach a constant figure before the specific activity had reached a maximum. With the assistance of Dr. Wm. Hauswirth of this laboratory a quantitative following of the fluorescent properties has begun. Fig. 1 shows the excitation and fluorescent curves of our better preparations. They are not identical with those of Minato & Werbin but they are not so very different. Although the curves in Fig. 1 are uncorrected, it appears that the excitation maximum is at a shorter wave length than that seen by the Texas workers. The fluorescent maximum ^{was} ~~were~~ the same (470nm) but the fluorescent curve in Fig. 1 falls off more symmetrically than does that of Minato & Werbin ('71).

FIGURE I

Fluorescence Spectra (uncorr.) of Purified Photoreactivating Enzyme from Yeast

Emission excited at 360nm

Excitation monitored at 470nm

4 μ g/ml of Protein

Sp.Act. EU/mg Protein = 2×10^6

The data in Table I shows that the EU/FI of fractions 4 and 7 are not very different though there is a 10-fold rise in EU/Pr. If the EU/FI increases beyond the figure of 6 ± 3 , as new procedures are applied and as the data in fractions 5 & 6 suggest is a possibility, it will mean either that inactive enzyme is being removed or that the fluorescence is not a critical part of the enzyme.

III. ENZYME COFACTOR

Experiments designed to demonstrate the presence of a dialyzable cofactor in the purified enzyme preparation seemed encouraging, at first. Dialysis against low concentrations of buffer was accompanied by a loss in enzymatic activity and fluorescence and this loss seemed to be restored by concentrates of the dialysate. However when adequate SH compounds were present the enzyme activity failed to fall below about 50 per cent and restoration with concentrates of the dialysate were irregular.

In an attempt to increase these differences, so greater reliance could be placed on the results, the time of dialysis, pH and nature of the buffers were varied but they failed to improve the conditions. At pH 3 or below the loss of enzymatic activity was greater but dialysates failed to restore the loss.

These results do not fit the picture of a simple dissociable protein-cofactor complex, especially the half active complex.

It is conceivable that the enzyme is made up of two types of such complexes with different dissociation constants. However, since the present assay for cofactor depends on the difference between 50 and 100% enzymatic activities each of which is subject to variations of about 20 per cent, it seemed unwise to put more than a minor effort in this direction especially since the enzyme purification is now going well.

A return to this problem will be swift if we obtain evidence of its existence and that changes in activity are great enough to be reliable.

RESEARCH PROGRAM OF DR. JOHN SCOCCASpecificity of DNA Uptake

In last year's report a preliminary view of the specificity of DNA uptake by competent H. influenzae was described. This work has been completed and a manuscript of a paper on this subject is attached. The results are summarized as follows:

Cells of H. influenzae strain Rd competent for genetic transformation bound irreversibly approximately 5 molecular fragments of H. influenzae DNA per cell. Under identical conditions DNA derived from E. coli B was not taken up (< 1 molecule per 10^2 cells). Similarly, DNA from Xenopus laevis was not taken up by competent H. influenzae. None of the heterologous DNA's tested interfered with the uptake of H. influenzae DNA as judged by competitor experiments employing either DNA binding or genetic transformation as test system.

Breakdown of heterologous DNA did not occur to any appreciable extent upon exposure to competent H. influenzae.

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