

Cohn

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28. RUE DU D^r ROUX - PARIS XV^e

TEL SEGR 01-10

PARIS, le February 27, 1951

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. Dear Dr. Lederberg,

We very much appreciate your recent letter and are sorry that there has been a misunderstanding between us. I don't feel it necessary to dwell upon the various aspects of this episode, more important being that we manage to keep in contact on our respective doings.

Thanks for reading the manuscript so carefully. I've introduced the corrections you suggested. We, too, have found that TEA has an inhibitory effect on the ONPGase activity but it isn't competitive with the alkali ions. On the other hand (table III) varying the TEA.PO₄ concentration doesn't affect the lactase activity. However, (table III), if TEA.PO₄ concentration is varied in presence of an activator K⁺ the activator goes through a maximum (significant although slight) which is not clear to us although we offer an explanation at top page 9.

I have decided to send you a rather detailed outline of our present work and findings so that you can have a comprehensive idea of ~~our work~~ ^{our} redirection of work.

This is a summary only of the recent work of our group. Roughly it can be divided into three parts.

I.- Isolation and characterization of β -galactosidase.

a) Purification.

Starting with kilo batches of acetone dried adapted ML E.coli we have isolated enzyme preparations with about 65,000 μ M/hr/mg N ONPGase activity (M/20 triethanolamine cacodylate pH 7.0,

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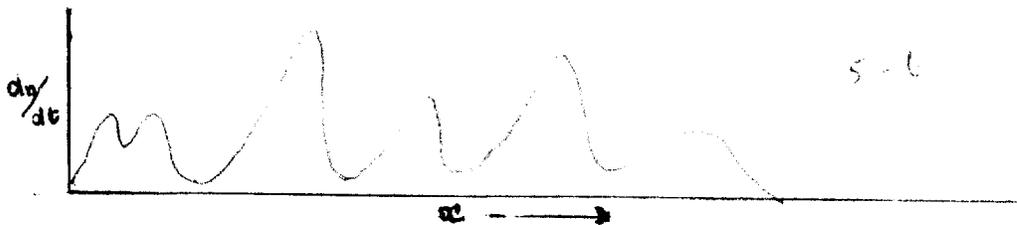
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M/10 NaCl M/600 ONPG, 28°C). The two key steps in the purification are 1) precipitation (at 0°C pH 7 M/10 K phosphate buffer) of unrelated impurities by saturation with butanol. The enzyme remains untouched in solution and there is almost quantitative removal of nucleic acids, polysaccharide and other gummy substances with a purification of 6-7 fold. The enzyme preparation in 1% solution is colorless which is esthetically if not chemically meaningful and the ratio of absorptions ^{at 280 mμ} drops to about 0.6. 2) taking advantage of the fact that the β galactosidase is more insoluble at room temperature than 0°C. If the butanol treated solution is brought to 35% saturated $(\text{NH}_4)_2\text{SO}_4$ at 0°C resulting precipitate removed and the supernatant then warmed slowly to 20-25°C. a second precipitate forms which is highly active.

We have used acetone, methanol, ethanol, dioxane precipitation with some purification regardless of solvent but none compares with butanol, at least in our hands. Elution and absorption techniques aren't very useful either.

b) Physical characterization.

In the ultracentrifuge our best preparations are still quite heterogeneous but ~~are a monomeric unit~~. Four or five ~~distinct~~ separate peaks each of itself quite homogeneous ranging in sedimentation constant between 4 for the lightest to 15-20 for the heaviest



typical sedimentation diagram of preparation 65,000 $\mu\text{M}/\text{hr}/\text{mg N}$

We too have carried out extensive electrophoresis experiments on paper and have evidence that in crude extracts the enzyme is complexed with other proteins which complexes are absorbed to the paper. We have completed quantitative electrophoresis experiments where the migration was carried out on double sheets of paper. One was developed colorimetrically by the Tiselius technic, the other, on the basis of the results with the first, cut into small uniform strips of 5-10 mms. Each strip was put into a 1.0 cc of NaCl 0.15 M and let stand overnight in the icebox. After the extraction

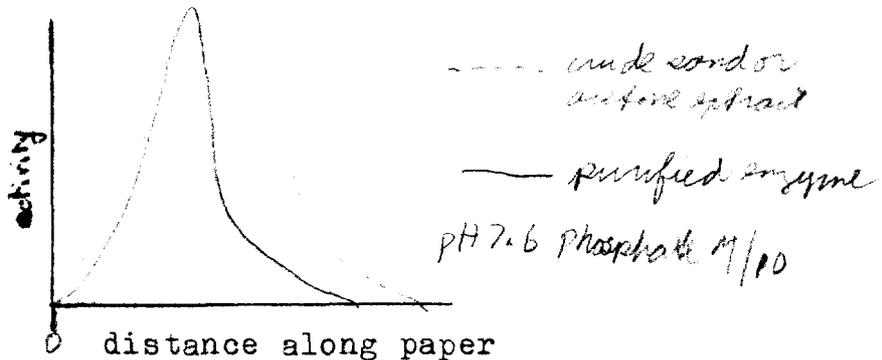
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of the enzyme, activities were determined and distance along the paper plotted against activity. For crude lactase there is consi-



derable spreading and apparent electrophoretic heterogeneity. The very best preparations have almost uniform Gaussian distribution. We are trying to distinguish between heterogeneity due to an artefact of fractionation and an actual heterogeneity in the synthesis of the enzyme. Our accumulating physical data indicates the latter but further work must be carried out.

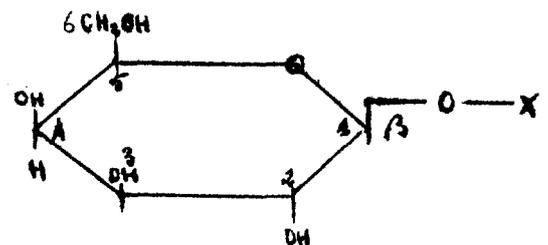
In addition we have completed some solubility phase rule studies on the enzyme but the work is as yet preliminary and seems least satisfactory as far as demonstrating heterogeneity is concerned because electrophoretically and ultracentrifugally complex preparations behave as single components in solubility tests ; a fact which may prove very significant.

c) Now to some characteristics of the enzyme ^{activity} itself.

Unfortunately, unable to get the necessary substrates by letter writing, Jacques and I visited Bell's lab in Cambridge and synthesized a whole series of galactose derivatives.

After testing a huge series of galactose derivatives we came to the conclusion that any change in the galactose ring structure destroys the affinity of the substance for the isolated enzyme. We tested for example the following substances.

At C ₆	COOH	galacturonic acid
	CH ₃	d-fucose
	CH ₂ OCH ₃	6 methyl
	H	arabinose
	CH ₂ NO ₂	6 nitro



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At C₄ inversion H/OH glucose
methylation
anhydro between 3 and 4
nitration

C₃ methylation
reduction-desoxy at C₃
nitration

C₂ same as C₃

C₁ α forms.

C1-C6 galactoson

We now have chloro derivatives and desoxy at C₂ which we haven't tried as yet.

On the other hand, as you know, any substitution at X leaves the activity as a substrate. For example we tested β methyl, β butyl, β phenyl, β naphthol, β nitrophenol, lactose, lactobionic acid, β para nitrophenol lactose. We now have β mannose galactoside and are synthesizing a $\beta\beta$ digalactoside which should have the highest affinity for the enzyme. It will probably interest Seidman and Link to know that we synthesized ONPG by a slightly different technic before knowing of their work, and confirmed their characterization of the compound.

Jacques studied the role of the ions with this battery of substrates and developed what he calls the "finger print" of the enzyme. By a combination of the various alkali ions with several substrates at different concentrations a series of K_s on V_m constants can be calculated which are reproducible with different enzyme preparations with extraordinary precision. This delicate test showed that during fractionation an alteration occurred in the structure of the enzyme which would not be revealable by activity measurements. On the other hand, it showed that the K_{12} and ML lactases were identical and also that the basal activity¹² is due to the same enzyme. We have begun a program of comparing the lactases of Aerobacter, Saccharomyces, Shigella sonnei, several strains of coli, L. bulgaricus, emulsian, megaterium and calf intestinal lactase.

In addition we synthesized a rather interesting substance, thiophenol- β -galactoside (TPG) which is not hydrolyzed by the enzyme but is a potent competitive inhibitor. I will talk about this later. We plan to make a whole series of thio compounds.

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We have also completed a rather striking experiment showing the competition between H^+ ions and Na^+ ions by studying the apparent affinity of Na^+ for the enzyme at various pHs between 5.0 and 9. As the H^+ concentration increases the affinity Na^+ decreases. It confirms the proposed theory on H^+ competitions.

Another interesting property of the enzyme is this. If complexone (Sequestrene binds all bivalent metals) is added to the enzyme it loses all activity (even the basal activity). Only Mg^{++} and Mn^{++} restore the activity, Fe^{++} , Ca^{++} , Ba^{++} , Sr^{++} , being without activity. ~~There are two effects here which we are experimentally separating, action specifically on activity and action of metal as a stabilizer of the enzyme against denaturation.~~

II.- Kinetic studies.

Jacques is completing a long series of kinetic studies on β -galactosidase synthesis. He used a mutant $M^+L^+G^-$ which cannot utilize galactose but in its presence forms the enzyme. Thus a separation of the substrate as an energy source and inducer is effected when maltose is energy source and non-utilized inducer ie. galactose is used.

Two technics were developed :

- 1) continuous culture in bactogen.
- 2) Priming technic. A culture is limited on maltose. When growth stops, inducer is added. No enzyme synthesis is noted until maltose is added. Immediately after addition energy source enzyme synthesis begins and the rate is linear from point 0.* This technic seems to remove the problem of penetration and is extremely useful.

Jacques has begun to compare the inductivity of the series of substrates and has some very interesting results. There is no evidence of autocatalytic synthesis and certain inducers for example β methyl galactoside, are active at a concentration of 10^{-6} by weight. His kinetics coupled with Pollocks on penicillinase, rule out quite thoroughly the autocatalytic synthesis at least in these two systems where defined enzymes are involved.

The whole series of galactose derivatives mentioned earlier were tested as inducers and again any substance which lost affinity

*. The reaction is stopped by addition of toluene after ~~10~~ 10 minutes following the addition of energy source. K12 adapts perfectly well so that I don't think you have to be envious of Stoner's enzymes. The activity is read in Putman with NPL as usual.

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for the enzyme also lost ability to induce. The intact galactose ring is necessary to the inducer. However the thiophenyl galactoside (TPG) although it has high affinity for the enzyme does not induce. This of course rules out the stabilization hypothesis. The TPG however inhibits competitively the induction by other substances and also inhibits growth on lactose showing that it penetrates. Your observation that neolactose is a substrate for the enzyme but doesn't induce it is extremely interesting. We would very much like to test this compound in our ML system. I wrote some months back to Richtmyer but he told me he had none left. Could you spare 20-50 mgs for us to test as inducer in our system? The TPG seems to inhibit the synthesis of basal activity. Your constitutive lactase seems to be a perfect test for the hypothesis of a natural inducer. The fact that glucose grown cells are optimally adapted to ONPG and not optimally to galactose is no evidence against the above hypothesis because the paths of galactose utilization as energy source and inducer might be different or because the level of enzymatic activity necessary to synthesize sufficient inducer to maintain a constitutive lactase is certainly much lower than necessary for optimal growth of cells. We still would like to try the TPG effect on basal enzyme synthesis if you're willing to send us this mutant.

III.- Immunochemical analysis of adaptation.

I can only here summarize the results obtained.

a) A rabbit antigalactosidase was prepared using the highly purified preparations. The serum precipitates but does not inhibit activity. The resuspended specific precipitate has full activity. A correspondance between the enzyme Gz as an antigen and as an enzyme can be defined in terms of enzymatic activity precipitable by unit quantity of antiserum. This antiserum doesn't affect amyloamylase.

b) There is present in unadapted cells a protein Pz which cross reacts to a large extent with the Gz.

c) Thanks to a peculiar immunochemical difference in affinities of the two proteins we were able to demonstrate that Pz was also present in adapted cells.

d) When the cells adapt to synthesize Gz the level of Pz falls to between 30-40 % of the original value indicating the Pz

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is converted to Gz. The effect is specific in that any adaptation not involving a synthesis of Gz doesn't affect Pz levels. This result has been repeated in a whole series of different mutants including KI2.

e) The Gz of all mutants tested, including KI2 and ML are antigenically and by "finger print" the same except one which needs further verification, a mutant analogous to your Lac1. *this mutant produces Gz only in response to alpha galactosidase.*

f) The Pz is present in all mutants of ML tested, L, G, M⁻ glucose⁻, etc, and is antigenically the same in all including KI2. We are starting a survey to study its distribution in bacteria (Shigella, Salmonella, etc).

g) The antiserum can be absorbed with Pz ~~or~~ (unadapted extracts) to leave a fraction which reacts only with Gz. Very recently, such that the ink hasn't dried ⁱⁿ our notebooks, we found that cells grown on maltose as energy source in the presence of TPG produce an antigen which precipitates the absorbed serum but which has no galactosidase activity. It seems as though TPG induces the formation of the "wrong" structure and if verified, will demonstrate that the combination of the ^{actual} inducer is not with the enzyme but with the precursor.

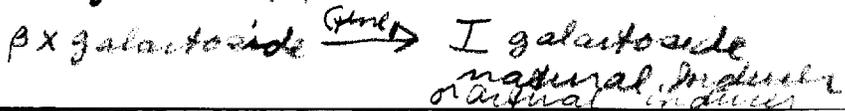
h) We have carried out extensive purification of Pz and it fractionates identically with Gz. In fact our best preparations of Pz have high activities of Gz due to ^{the} concentration of the trace basal activity. We are beginning some immunochemical analysis on enzyme. In addition we are preparing anti Pz sera to more directly study this protein. *This is difficult because Pz is poorly antigenic.*

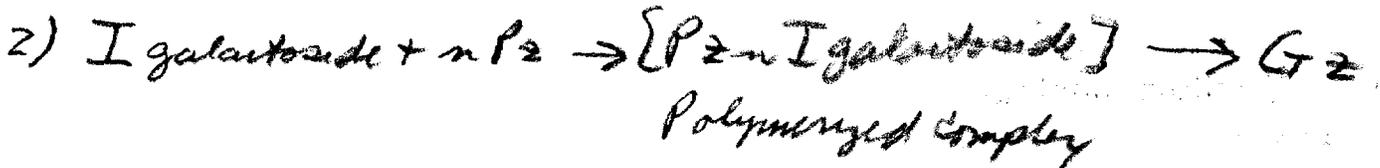
Our total work to date has led us to a hypothesis that the precursor Pz is converted to Gz by polymerization around the inducing substrate*. The kinetics, the immunology and other circumstantial data including the heterogeneity suggest this hypothesis. In any case it is a good working assumption and we hope before not too long to have the molecular weights of Pz and Gz.

Dr. Wainwright from England is with us now, working on the amyloamylase along the same lines that we've carried out with the lactase and has purified that enzyme quite considerably.

I have outlined our work in considerable detail in the hope that our laboratories can from now on maintain a close correspondence. *We* feel that further duplication of work at this stage of

** I should enlarge this a bit by describing the theory in formula.*





Three theory products

- 1) The molec. wtd. of Gz will be higher than Pz
- 2) That there is a natural inducer synthesized from gene which combines with Pz to give enzyme. Possibly steps (1) & (2) are identical & i.e. are one.
- 3) The specificity of the induction by external inducer depends on whether the gene can catalyze the conversion to natural inducer.

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our development would be unfortunate in view of the fact that there are many aspects of the problem we are letting fall only because unwittingly we are repeating the efforts of others. Our efforts have been directed toward defining the biochemical aspects of the system. We are particularly interested in the mechanism of synthesis of adaptive enzymes and have characterized and isolated the enzymes and their possible precursors only because it was a necessary preliminary to our work. We have a huge gap in our facilities to study the genetic aspects of this problem even though the presence of Francis and Betty Ryan has given us a shot in the arm.

You mention in your letter a desire to discuss a planning of the lines of work which should be followed in the different laboratories. We would like to offer some sort of collaboration but the distance between us might be a deterrent. In any case we would be delighted to characterize for you any of your mutants as regards properties of enzyme (finger print), specificity and rates of adaptation, antigenic properties of Gz and Pz. We are not in any way prepared to handle the genetics and we have developed quite extensively the biochemistry, so that it seems as though we could have a profitable exchange of materials. We would like to continue to develop our present lines of work without entering into competition with anyone and we can do this by keeping in close touch with you. We will send you any and all manuscripts before publication.

I plan a rather extended stay in France. I find this country rich in human and cultural values which is not lacking in the States are at least different. The lab itself is an extremely inspiring place to work in and we have coming to work with us from the States a very wonderful group of people including the Doudoroffs and Ryans. I am glad you are in contact with Alain. My regards to him. If you see Dr. Williams and Dr. Phil Cohen around send them very best. Deutsch will stay with us several weeks in July before returning to the States.

Thanks again for your letter.

Sincerely yours,



Melvin COHN

Sorry to have worked over this with a pencil but I don't have the heart to ask our secy. to retype it.