Dump Talk

Chemostat

~~other methods Beadle Brooks~~

Transformation

nucleic acid polymer

Caffeine

Guanosine

Beadle ~ Biochem pathways || one gene one enzyme

Watson Crick

Inductive enzymes

Lactose glucose - galactose

T.M.G. thio methyl ||

Chemostats maximum -

1)

2. Epigenic concept of enzyme

regulation I

II

In talking to you I shall limit myself to the area of microbiology and the only organisms I shall speak to you about will be two bacteria; namely, pneumoccoci, which due to the judicious use of penicillin may soon be extinct, and the bacterium, coli, which is the most common inhabitant of the intestines of mammals. I shall speak also of viruses, but only of one class of viruses, which are called T-phages, and which can infect the bacterium, coli. If you mix in a testube a culture of bacteria with a suspension of one of these viruses, a virus particle will be absorbed (adsorbed ?) to a bacterium, and after about 20 or 30 minutes, the bacterium will burst and out will come about a 100 or 200 virus particles. When coli grows and multiplies, when a strain of coli which is in this sense sensitive to the T-virus which we use occasionally a mutant bacterium arises which is not attacked by the virus. A mutant to virus resistance is a change in a character of the bacterium which is inherited by its offspring. If you have a suspension of bacteria, it is very easy to discover in such a population the mutants which are resistant to the virus. All you have to do is add a drop of virus to a testtube which contains the bacteria, wait until the virus is adsorbed ?, and to this mere drop of the mixture on the surface of nutrient algar. The mutant bacteria, which are resistant to the virus, will grow into colonies, whereas the bulk of the bacteria population, which is sensitive to the virus, will be killed.

We do not really understand chemically what a mutation to phage resistance means, and the only reason we work with such mutations is the fact that it is so easy to count the number of mutants. Apart from this advantage, we much prefer to work with so-called biochemical mutants. What are biochemical mutants? Metabolites like amino acids are synthetized by the cell from simple compounds through a number of biochemical steps. Each such step is catalyzed by an enzyme. We imagine that each such enzyme is a specific protein molecule which has a specific sequence of amino acids, and that there is a gene which we believe to be a nucleic acid polymer, and that for each enzyme the cell contains a specific gene which determines somehow this specific sequence of amino acids. This is called the one-gene-one-enzyme hypothesis which was formulated by Beadle and Tatum, who were the first to work extensively with biochemical mutants. If the mutation occurs which renders the gene defective, then the corresponding enzyme is not functional, and accordingly there is one step in the biochemical pathway leading to the amino acid in which we are interested which the mutant cell cannot perform, and in these circumstances if one wants to grow the mutant cell one has to supply in the medium that particular acid which the cell is unable to synthetize. Why are we so sure that it is the nucleic acid polymers which determine what enzymes are synthetized by the cell? The best evidence for this is as follows:

If we take a strain of pneumoccoci, which is sensitive to streptomycin, we can change the bacteria which belong to this strain into bacteria which are resistant to streptomycin, and the offspring of these transformed bacteria will also be resistant to streptomycin. Now we can bring about this transformation by a related strain of bacteria which is resistant to streptomycin, extracting the nucleic acid from it, and when we mix this nucleic acid with a suspension of the streptomycin-susceptible strain and let it stand for

a while we find streptomycin-resistant bacteria, and we find that all the offspring of this resistant bacteria are also resistant. In order to prove that it is indeed the nucleic acid which has brought about this transformation, we must show that the nucleic acid extract loses its ability to bring about a transformation when treated with an enzyme that depolymerizes the nucleic acid. This is indeed the case, and so we can be sure that it is the nucleic acid and not some impurity in the extract that performs the miracle of transformation.

There are two kinds of nucleic acid in the bacteria which differ in the 5-carbon sugar that is contained in the polymer; the nucleic acid that contains an oxygen atom attached to the 2-carbon position of the sugar is called ribosenucleic acid, and the other nucleic acid which does not contain an oxygen atom in this position which is called desoxyribosenucleic acid, and it is easy to show that it is this latter which is responsible for the bacterial transformation we have observed.

It is still an unsolved mystery how this nucleic acid can do two things: reproduce itself and control the synthesis of the various enzymes.

We might be on the verge of understanding how nucleic acid reproduces itself. A few months ago, for one afternoon, I thought I understood also how a specific nucleic acid polymer can make a specific protein. It was a nice try but it did not pan out. I shall come back to ths point in a moment.

Now a nucleic acid polymer is built as is shown in Fig. 1. There is a sequence of purines or pyrimidines, and it is believed that this represents a code which is somehow read by the amino acids and that in a given sequence of purines and pyrimidines there corresponds a sequence of amino acids. The purine-pyrimidine bases are tied together with five carbon sugars and the adjacent five-carbon sugars are linked to each other by means of phosphate groups. The actual structure of the desoxyribosenucleic acid, which was postulated a few years ago by Watson and Crick, you can see from the second figure. It is believed that two strands of nucleic acids form a double stranded helix and that the purine-pyrimidine bases of one strand are attached by hydrogen bonds to the purine-pyrimidine bases of the other strand. The next figure, 3, will make this a little more clear.

People believed that such a nucleic acid can reproduce itself by separating into two single strands, and that alongside of each strand is then form the complementary strand of nucleic acid so that in place of one double helix we now have two double helices. How can such a nucleic acid lead to the formation of a specific sequence of amino acids? It seems clear that this cannot be the work of simple chemical forces which act between a single strand of nucleic acid and the amino acid molecules which may freely diffuse around within the cell. The theory for the synthesis of proteins which I attempted to form but which did not pan out was based on a rather different concept. One may assume that there are in the cell twenty different enzymes, and that each of these twenty enzymes catalyzes the formation of a specific trinucleotide carrying one specific amino acid. The purine-pyrimidine

bases of these trinucleotides could then pair with the complementary bases along a single stranded nucleic acid polymer, and thereby line up different amino acids in a sequence which is determined by the sequence of the purine-pyrimidine bases along the nucleic acid chain. The trouble with this picture is the fact that the distances between the amino acids when they are lined up in this manner are much greater than the distances of adjacent amino acids in a polypeptide. Drs. Crick and Brenner in the Cavendish Laboratory at Cambridge are hard at work to find a way out of this delimena. If one seriously believes that the genes are composed of nucleic acid polymers, then one might think that it should be possible to damage the genes in a growing bacterium by exposing the growing bacterium to chemical analogues of purines or pyrimidines, and thus obtain mutations.

I must now say a few words about a new technique which Dr. Novick and I developed for the purpose of studying mutations and some other basic phenomena in growing bacterial cultures. We developed a device which we call the "Chemostat" which opened up a new way of studying such phenomena in bacteria. Fig. 4 shows the schematic view of this device. You see here a tank which contains a nutrient liquid, and a few drops of this nutrient liquid are allowed to flow into the growth tube where there is maintained a suspension of growing bacteria. In many of these experiments we used a bacterium which required tryptophane as a growth factor because, in the absence of a certain functional enzyme, this bacterium can make its own tryptophane. When this strain is grown in the Chemostat, the tryptophane concentration in the tank determines the population density of the bacteria in the growth tube in the steady state, and the rate at which we allow the drops to fall into the growth tube determines the rate at which these bacteria can synthetize their proteins. Thus, by regulating the rate of flow, we can regulate the growth rate of the bacteria or, if you wish, the length of the generation time. In this manner, we can vary the generation between a generation time of two hours and a generation time of twelve hours.

The population in the growth tube represents a stationary state in all respects except one. Mutations occur in such a growing population. For instance, if we grow a strain which is sensitive to a certain virus, which we call T-5, there will arise bacteria which are resistant to T-5, and the offspring of such bacteria is again resistant to T-5. Because of this, the number of virus mutations resistant to this virus is not constant,but it increases linearly with time. We can maintain such a population growing in the Chemostat for many weeks or months, and each day we can determine the number of mutants which are resistant to T-5 by plaiting a drop of the suspension together with a drop of virus T-5, allowing colonies to grow up on an agar surface, and then counting the number of colonies. Each of these colonies arises from a single mutant bacterium, and the non-mutant bacteria which are sensitive to the virus do not yield any colonies. The curves shown in the next figure are obtained in this manner.

We had expected to find that whether we grow the bacteria slowly or fast, we will get the same number of mutants produced per cell division. This, however, is not case; quite the contrary. What remains constant is not the number of mutants produced per cell division but the number of mutants produced per unit time.

What happens now if we grow bacteria in the presence of a chemical analogue of purine or pyrimidine which is a constituent of the nucleic acid. In the next figure we see the structure of a few such chemical analogues. One of the compounds we used was caffeine. The next figure shows what happens when we add at a certain time caffeine to the growing culture. To produce a ten to twenty-fold increase in the mutation rate, we must use about 100 milligrams per liter of caffeine in the nutrient liquid. Some of the other purine analogues act very similarly to caffeine. Here we have to deal with a class of compounds which causes mutations without causing any appreciable amount of

killing, and in this respect this class of compounds is almost unique. Apart from studying compounds of this sort which we may call mutagenes, we also discovered a class of compounds for which there is no precedent. These compounds which we call antimutagenes counter-affect all mutations of the purine type. One of these compounds is guanazine(?) which is, as you will perhaps remember, nothing but quanine to which is coupled a five-carbon sugar. Quanine is a constituent of nucleic acid. In order to counteract the effect of 100 milligrams per liter of guanine, all one needs is to add one milligram per liter of guanazine. You may see what happens when we add guanazine to a particular culture which grows in the presence of caffeine.

I am now going to leave this class of phenomana in order to talk a little about the second of the mysterious protein synthesis, and as you will see, again the Chemostat enables us to gain some insight into this phenomenon which could not have been obtained by the conventional methods of study. If you want to learn something about the synthesis of enzymes, you have a better chance of doing so if you study an enzyme, the synthesis of which can be turned on and turned off. There is a large class of enzyme where this can be done, and they are called 'adaptive enzymes.' One of the classical examples, and one which has been studied with great intensity in the last few years, particularly by the school of Jacques Monod at the Institute Pasteur in Paris, is the formation of lactase. There are many strains of bacterial coli which do not contain this enzyme in appreciable amounts. This enzyme is capable of splitting lactose, which is a disaccharide composed of glucose and galactose into its component monosaccharides. When these same bacteria are grown in the presence of lactose, they make at a rapid rate the enzyme lactase, and this enzyme may then be present in the bacteria in the amount of 10% of the total protein content of the bacteria. We say that lactose induces the formation of the enzyme. Certain chemical

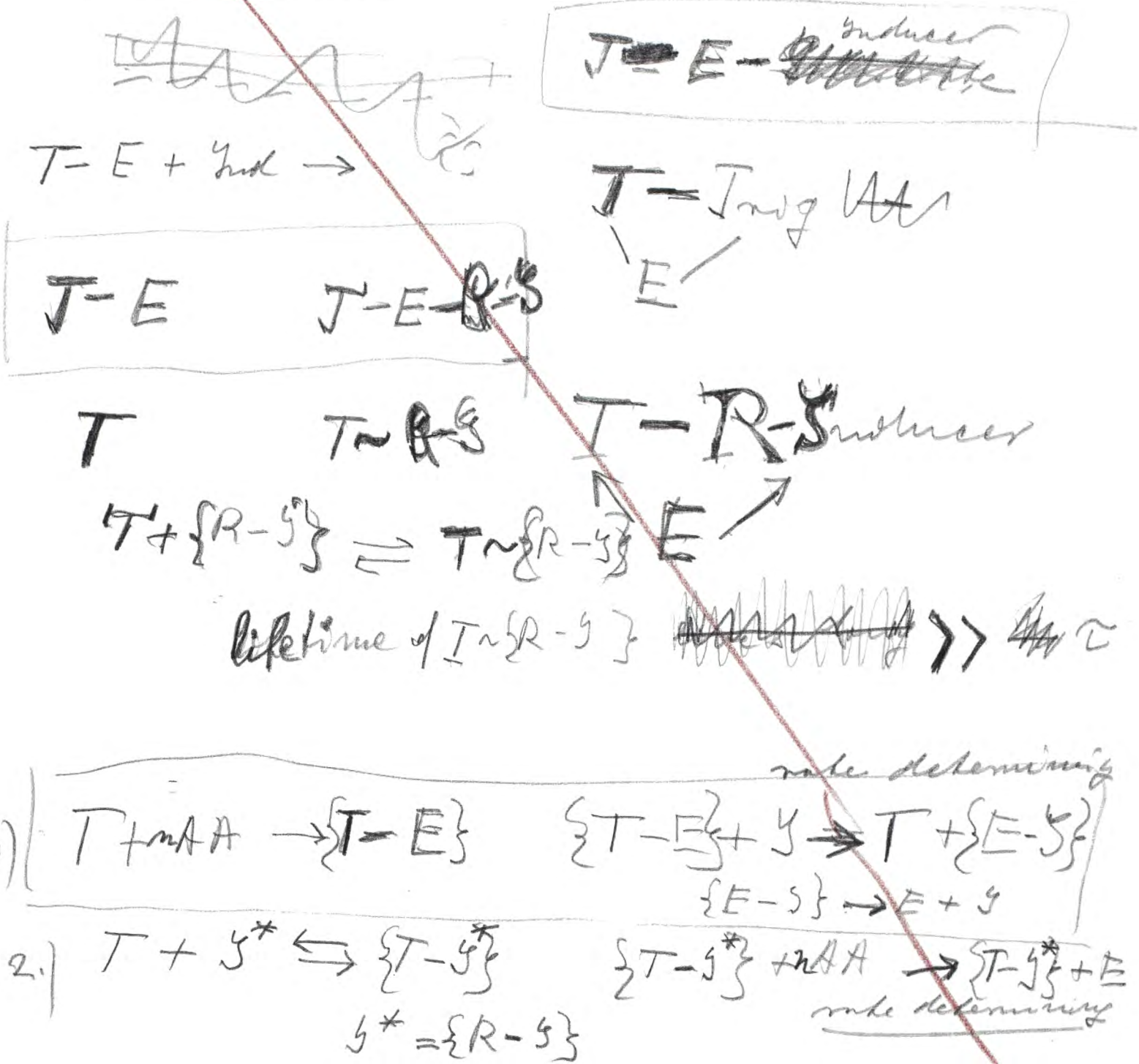
analogues are even better inducers and since, in contrast to lactose, they are not split by the enzyme, they are to be preferred in most experiments. One of these inducers is called TMG because it is a thiomethylgalactoside. The intensive experimentation with this system has so far not produced major insights primarily because the experimenters were tripped up by a truly devilish complication.

The next figure shows what happens when you add inducer to a culture of bacteria that grow in the Chemostat. You see there is a slow rise in the amount of enzyme per cell which goes over many generations linearly with time, and if you plot not the amount of enzyme per bacterium but the rate at which the enzyme is synthetized per bacterium, you get this dotted line. This observation blocked all progress until Dr. Aaron Novick and Dr. Milton Weiner working in Chicago described in a peper which appeared this year the explanation of this curious phenomenon. It turns out that what we are measuring here is not the amount of enzyme that is present in each bacterium, but rather that a small fraction of the bacteria contained no enzyme but rather that a small fraction are fully induced and contain a large amount of enzyme where the rest of the bacteria contain none, and the rise in the enzyme with time which we observed is due to the increase in the number of bacteria which are induced rather than to the increase in the amount of enzyme which each bacterium contains. The fact of the matter is that we are dealing here not just with one enzyme lactase which is induced by the inducer TMG that has penetrated into the bacterium but also we are dealing with an induction of a system which pumps the inducer from the nutrient solution through the cell wall into the bacterium. If you really want to study how the concentration of the inducer inside the bacterium controls the rate at which the bacterium makes the enzyme lactase, then we must work with mutant bacteria which have lost the ability to pump inducer from the nutrient medium through the cell wall into the bacterium. If we had that, we then

obtain a very different shaped curve which is shown in the next figure. Fig 2.

I ~~think one may be confident that now that we know how to best the devil~~ progress ~~in the study of enzyme induction will go very fast.~~

No kinetics —
1000 fold increase
no amplification of genes!
Two theories



In one generation time the enzyme per bacterium reaches 63% of the maximum, and this means that almost immediately, if not immediately, upon adding the inducer the enzyme is produced at a fixed rate. In this sense you might say that there is no such thing as kinetics of enzyme formation, and the hope of learning something about the kinetics of enzyme formation must be abandoned. This is almost true but not quite, as we shall see in a moment.

The enzyme lactase is present in a growing bacterial population in the absence of inducer but it is present in a very low level. In the presence of inducer, this level is raised about ten thousand-fold. The inducer might increase the number of paragenes ten thousand-fold which produces this particular enzyme. This is a notion which, I believe, most people would reject, or else we must say that the number of paragenes is not increased but the rate at which one paragene makes enzyme is increased ten thousand-fold. How could this be accomplished? Well, this could be accomplished in two very different ways.

Protein synthesis 8

No one seriously doubts today that in some manner the sequence of the purine bases along a nucleic acid strand determines the sequence of the amino acids in the corresponding enzyme. This does not necessarily mean that the gene itself makes the enzyme. There might be some sort of copy of the gene; perhaps the ribosenucleic acid rather than a desoxyribosenucleic acid which makes the enzyme. For the sake of simplicity, we shall simply call the unit which contains the same information as does the gene, and which may or may not be the gene itself that actually makes the enzyme, a paragene. Crick and his co-workers have suggested that the amino acid sequence is written into the nucleic acid strand of what I have just called a paragene in a code. In this code, each word means one of the 20 amino acids, and each word consists of three letters which are chosen from among the 4 purines and pyrimidines; quanine, etc..... Crick et al., in a letter published this year, have shown that such a 20-word code can be constructed on the basis of such three-letter words which requires no commas. The state that there are no commas means that you need not read the code from one end of the paragene onward; you can start reading it at any point on the paragene without having it indicated whether a given purine or pyrimidine is the first, second, or thrird letter of a code word. So far so good, but now comes the big question: how can the amino acids read the three-letter code words? There are no known chemical forces which would line up the amino acids in the proper sequence alongside such a nucleic acid, either single-stranded or double-stranded, polymer. Crick made very tentatively the suggestion that there are perhaps 20 enzymes in the cell, and each of these couples one specific amino acid with a specific sequence to the other nucleotides. One might imagine that the amino acid is coupled to a phosphate group on the first or the third nucleotide, forming an acid anhydride, either P or PP, bond which could very easily split 12,000 or 16,000 calories, respectively. These trinucleotides would be complementary to the code words; id est, quanine is replaced by ..... One can then

see how the amino acids might be lined up in the proper sequence alongside the nucleic acid strand through the pairing of the complementary bases of the trinucleotides with the bases contained on the paragrne. Each trinucleotide is reversibly combined with the proper code word on the paragene until something triggers a chemical reaction chain which splits the amino acid anhydride, and the energy liberated is utilized for the formation of a peptide bond between the adjacent amino acids. In this way a polypeptide may be formed with the amino acid sequence which is determined by the paragene. There is one great difficulty with this picture. When the amino acids are lined up alongside the paragene in this manner, then the amino acids are at a much greater distance from each other than the adjacent amino acids that are contained in a polypeptide. This is certainly so if the nucleic acid strand that represents the paragene has a configuration anywhere resembling the helical configuration of DNA. I have tried hard to overcome this difficulty and find some plausible structure for the paragene that is not encumbered with this difficulty, but I failed. Right now Crick and his co-workers are hopeful that they may be able to overcome this difficulty. Whether or not they shall succeed remains to be seen but in the meantime we shall assume that the general features of the picture which I gave you will be retained when we shall know the real solution to this problem. On this basis I made an estimate for the time it might take to form a polypeptide alonside a paragene, assuming that the triggering reaction which we mentioned before occurs very frequently. The rate-limiting factor then becomes the concentration of the amino acids. On the basis of the known facts concerning the rate of synthesis of enzymes which are very highly induced, one arrives for the time, T_{AA}, needed for the formation of the enzyme of about one second per paragene. At this point, we may just as well assume that there is one paragene present for each enzyme per bacterial cell.

A priori you might say that the first mechanism is far more likely because we know that lactose has a chemical affinity for lactase which must combine with it in order to split it, and there is no reason to believe that lactose should have any affinity for the template which is presumably a nucleic acid polymer. But you will see in a moment that we are practically forced to reject scheme 1 and to adopt scheme 2, Because we have no reason to believe that the inducer lactose, or such a chemical analogue of lactose as TMG, has any affinity for the template, we are led to believe that the inducer I^*

$$I + R \longrightarrow \{I - R\}$$

$$\downarrow$$

$$I^*$$

When we grow the bacteria in the chemostat at low generation time, and if we slow down, for instance, the rate at which the bacteria synthetize protein by controlling the rate of growth with tryptophane which the bacteria cannot make, then we should expect that the rate-limiting factor is a supplier of tryptophane and the rate at which tryptophane can be assembled on the paragene zipped together with other amino acids already waiting on the paragene into the polypeptide that represents the enzyme. If Equation 1 were the correct description of enzyme induction, we should then expect at high inducer concentration and a slow rate of protein synthesis to find less enzyme in the bacterium than at fast protein synthesis. This, however, is not the case, and I am, therefore, concluding that it is not scheme 1 but scheme 2 which gives us the true story of enzyme induction. I am led to this same conclusion also in another way. There exists not only a phenomenon called enzyme induction, but also a phenomenon called enzyme repression. To give you one typical example: In the biosynthetic pathway that leads to the synthesis of arginine

(Insert

- shift [illegible] with Arginine

In cases of this type, depending on the strain of bacteria used, one might find that by raising the arginine concentration very high, one can completely suppress the formation of the enzyme. Whereas in the cases of other strains, arginine does not suppress the formation of the enzyme but the rate of enzyme formation can be greatly enhanced by depressing the arginine level below that which is contained in the bacterium when it makes its own arginine. To show that, one must take a mutant which cannot make arginine and grow it in the Chemostat where the arginine concentration in the growth tube can be maintained exceedingly low if the flow rate is so adjusted that the generation time is increased perhaps up to five-fold. If we had to postulate a separate mechanism for the repressor of enzyme formation and a separate mechanism for the enhancement of enzyme formation by means of an inducer, this would be rather awkward, but I am inclined to believe that the scheme given under 2, to which we gave our preference just a few minutes ago, can account for the phenomena observed with raising or lowering the arginine concentration that we just mentioned. According to the notion here adopted, we would not regard arginine as a repressor but rather as a weak inducer. Arginine or more likely arginine* competes with stronger inducers which may be represented by the precursors of arginine, such as ornithine or citrulline. When we say that arginine is a weak inducer, we can in this context say that the rate of the reaction listed under 2b is low, but I* is represented by arginine*, and that it is higher when I* is represented by ornithine* or citrulline* . All these different inducers, I*, compete with each other for the template, T, and if the concentrations involved are sufficiently high and the equilibrium constants for the reaction, 2b, sufficiently low (i.e. if the binding energies are sufficiently high), then the arginine may very well appear to be a repressor.

The situation is, however, very different in the metabolic pathway in which an amino acid, for instance, tryptophane, (is not built up but) is degraded and modified.

Fig._____

If, indeed, ornithine* and citrulline* are stronger inducers than arginine*, this might not necessarily become manifest, for if we increase the concentration of ornithine and citrulline, these are in general converted into arginine, and presumably also into arginine*, and if the stationary state is such that concentration of the free template, T, is low, concentration to the complex templates T-I*, then enzyme production will not be appreciably enhanced upon raising the concentration of citrulline or ornithine. As a matter of fact, no case is known to me where a precursor of an amino acid manifests itself as an inducer of any of the enzymes that lead from this precursor to the amino acid. ~~On the optimistic assumption that we have indeed succeeded in guessing at the correct mechanism of enzyme production-repression, there remains a major mystery about the inducible enzymes!~~ In contrast to precursors of amino acids which do not manifest themselves as inducers, even though they may well be, we have ~~a large class of compounds~~ which are inducers of the enzymes which carry the metabolite one step forward on some metabolic pathway. One might almost say that an enzyme which produces a metabolite is, in general, produced by the precursor of that metabolite, provided only that we are not on the biochemical pathway which leads to the formation of an amino acid but rather on a metabolic pathway which degrades or otherwise modifies an amino acid.

Fig._____

On the basis of the notions here developed, we may say that while a precursor may be a good inducer of the metabolite to which it is converted .....may be a weak inducer, the precursor manifests itself as an inducer for either of two reasons:

A. It might be that the concentrations and equilibrium constants are such that most of the template in question is free and that only a small fraction is combined with the various I*'s correspond-

ing to the precursor of the metabolites which are produced from it. In the case of these metabolites which are weak inducers they cannot suppress.

B. It might be that the compound precursor-R, which is present in the metabolized bacterium, even in the absence of the precursor, I, is converted by the enzyme, E, into the weak inducer, a metabolite-R. When the precursor is now added to the medium in which the bacterium grows, it competes with the enzyme, E, for the compound, precursor-R, and thereby depresses the formation of the weak inducer, metabolite-R.

This does not resolve what may