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Liam Robert Butland Mann
(Ph.D. Thesis)
August 1969

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CHEMICAL EFFECTS OF D$_2$O ON ESCHERICHIA COLI

Liam Robert Butland Mann

Laboratory of Chemical Biodynamics
Lawrence Radiation Laboratory
University of California,
Berkeley
August, 1969

ABSTRACT

Growth of E. coli is inhibited by >90% D$_2$O, but it can be gradually trained to grow in 99.5% D$_2$O.

1. Steady States. In minimal-acetate(d$_3$)-99.5% D$_2$O medium (abbreviated to all-D) the mass doubling time after 20 or 200 generations is 5 – 6 hrs, cf. 2 – 2.5 hrs in a medium which is identical except for being unenriched in deuterium (abbreviated to all-H). The protein/DNA ratio after adaptation to all-D is double the steady-state value in all-H. This is qualitatively as predicted by Orgel (J. Mol. Biol. 9, 208, 1964) from the hypothesis that de-repression of many enzymes will occur to compensate for lower turnover numbers in D$_2$O. D-adapted mutants of the lac operon (i$^+$, i$^-$ and o$^c$) give the same differential rates of synthesis of β-galactosidase (BG) as in all-H. The turnover number under the standard conditions of BG assay was measured on BG purified by polyacrylamide-gel electrophoresis (PAGE) and did not differ significantly between BG samples made in the two media. The kinetics early in induction of BG are the same in all-D as in all-H; since the lag period between addition of inducer and the first appearance of induced BG
is known to reflect mainly the time required to synthesize lac mRNA, this suggests that transcription is not a main site of inhibition of protein synthesis in the all-D steady state. The D-adapted cells are more sensitive to induction by sub-maximal concentrations of inducer. This may mean either that the lac repressor binds the inducer more tightly in the all-D cells, or that they make less lac repressor. Soluble protein profiles on PGE differ between the all-D and all-H steady states.

2. Isotopic Shifts. BC-forming potential induced in H-adapted cells is translated only half as efficiently in inducer-free all-D as in inducer-free all-H. This suggests that for BC synthesis translation is inhibited by D$_2$O. However, when induced D-adapted cells are shifted into all-H containing inducer there is a repression similar to catabolite repression. Therefore, the inhibition of translation of BC mRNA in such an isotopic shift may be the result of catabolite repression and thus may not be relevant to the synthesis of most proteins. Rates of synthesis of DNA, RNA and protein also show dramatic changes after D-adapted cells are shifted into all-H. Growth is prompt and fast. RNA synthesis speeds up five-fold and then slows within a generation. Protein synthesis accelerates tenfold. DNA synthesis, however, does not accelerate for a generation and then occurs in a burst, as previously reported for similar shifts with Bacillus subtilis (Yoshikawa and Sueoka, Proc. Nat. Acad. Sci. 49, 806, 1963). Two generations of growth back in H$_2$O destroys the ability to grow in D$_2$O without re-training.
3. **Long-term Changes.** Within ca. 100 generations in all-D, two \(i^+\) and two \(o^c\) mutants lost most or all ability to make BG activity. In one of the \(i^+\) strains this was shown by PGE of soluble proteins to be due to inactivity of BG rather than failure to make the BG protein. The loss occurred only in all-D. It is stable over many generations in all-D, or in all-H (a medium which will not bring about the change), but is reversed by glycerol and is therefore not a mutation.

**Conclusion.** The Orgel hypothesis is qualitatively satisfactory, but protein synthesis does not quantitatively compensate for enzyme inhibitions in \(D_2O\). Experiments on the synthesis of one protein, BG, suggest that it is at translation and not transcription that protein synthesis is inhibited in \(D_2O\). Ability to grow in \(D_2O\) appears to consist of some particular balanced macromolecular composition, including a higher protein/DNA ratio and different distribution of proteins, which the cell can achieve (given time through gradual training) by de-repressing some enzymes.
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I. INTRODUCTION

A. Biological Effects of Deuterium

Soon after highly-purified D$_2$O became available, G. N. Lewis discovered that tobacco seeds will not germinate in it. Since then scores of papers have described biological effects of D$_2$O. Thomson's fairly recent monograph on the subject gives summaries and discussions of many such experiments. In higher organisms, D$_2$O is often lethal. For example, rats drinking 50% D$_2$O die when their body water reaches only 30% D$_2$O (in a month or so). Some very lowly organisms can adapt to >99% D$_2$O, but even these always show disorders such as slow growth. On the molecular level, almost nothing is known about how D$_2$O works as a poison.

B. Possible Explanations

Table I compares some selected properties of H$_2$O and D$_2$O. Such a comparison can be useful, but obviously not in the sense of using it in any simple way to explain which biological effects D$_2$O causes, or even why there should be any effects at all.

Since almost all biochemical reactions are enzyme-catalyzed, predictions and explanations of biological effects of D$_2$O must focus mainly on possible effects of D$_2$O on enzymes' catalytic effects. One can even imagine qualitative changes caused by D$_2$O; for example,
Table I. Comparison of selected properties of D$_2$O and H$_2$O (ref. 35).

<table>
<thead>
<tr>
<th>Property</th>
<th>D$_2$O</th>
<th>H$_2$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific gravity at 25°</td>
<td>1.1078</td>
<td>1 (defn.)</td>
</tr>
<tr>
<td>Temp. of maximum density</td>
<td>11.2°</td>
<td>3.98°</td>
</tr>
<tr>
<td>Melting point</td>
<td>3.8°</td>
<td>0.00°</td>
</tr>
<tr>
<td>Boiling point</td>
<td>101.4°</td>
<td>100.0°</td>
</tr>
<tr>
<td>Viscosity at 5°</td>
<td>19.9 millipoise</td>
<td>15.2</td>
</tr>
<tr>
<td>Viscosity at 35°</td>
<td>8.64</td>
<td>7.21</td>
</tr>
<tr>
<td>Surface tension at 25°</td>
<td>same within 0.05%</td>
<td></td>
</tr>
<tr>
<td>Dielectric constant at 25°</td>
<td>78.25</td>
<td>78.54</td>
</tr>
<tr>
<td>Ionization constant at 25°</td>
<td>$1.95 \times 10^{-15}$ M</td>
<td>$1.0 \times 10^{-14}$ M</td>
</tr>
<tr>
<td>Relative solubility of KCl at 30° (molar basis)</td>
<td>93%</td>
<td>100%</td>
</tr>
<tr>
<td>Zero-point energy</td>
<td>9.66 Kcal/mole</td>
<td>13.22</td>
</tr>
</tbody>
</table>

The recognition of codons by tRNA might show random or systematic errors, leading to different primary structures in the proteins synthesized. More likely, however, are quantitative effects on rates of enzymatic reactions. The following reasons for changes in an enzymatic reaction's rate-constant are possible: $^{63}$ (1) primary isotope effects due to deuteration of substrates, (2) secondary isotope effects, (3) solvent effects on metal co-factors, dissociation of weak electrolytes, and diffusion coefficients, and (4) effects on the enzyme's structure. All these might change an enzyme's turnover.
number. Such effects might also, indirectly, change reaction rates by changing the concentrations of substrate and enzyme molecules \textit{in vivo}. Then, too, there might be changes in permeabilities of membranes.

Several studies have been made\textsuperscript{63} of D\textsubscript{2}O's effects on enzymatic reactions \textit{in vitro}. Equilibrium constants have almost always been assumed not to change to any extent of biological significance. The equilibrium constant for the addition of water to fumarate, catalyzed by fumarase, is 5-7\% lower in >99\% D\textsubscript{2}O (ref. 1). As for the kinetic isotope effects, k\textsubscript{H}/k\textsubscript{D}, theoretical maximum values at about 30° are 7, 8 and 10 for reactions in which rate-limiting steps break C-H, N-H and O-H bonds respectively,\textsuperscript{67} but typical experimental values for enzymatic reactions \textit{in vitro}\textsuperscript{63} have been in the range 1-3. Nearly all these experiments used the normal enzyme (i.e. isolated from an all-protiated organism) and protiated substrates, so that the k\textsubscript{H}/k\textsubscript{D} values measured were, in a wide sense, only solvent effects. The one experiment most relevant to my work is that of Rittenberg and Borek.\textsuperscript{58} They grew \textit{E. coli} (strain B) in a minimal medium made from 99.5\% D\textsubscript{2}O and containing deuterioacetate as sole carbon source. Presuming that deuterium was not strongly discriminated against, after many generations at least 99\% of the hydrogen in the cellular compounds must have been deuterium, including those non-exchangeable hydrogen atoms bonded to carbon, as well as the exchangeable ones bonded to O, N and S. From these fully-deuterated cells was made what the authors called an enzyme (it was, in fact, a crude mixture of enzymes). This preparation
was capable of catalyzing oxidation of succinate by added ferricyanide. The rate of reduction of ferricyanide was \textit{the same} whether tetradeuteriosuccinate or ordinary succinate was supplied, whereas the corresponding system from non-deuterated cells could oxidize tetradeuteriosuccinate only half as fast as ordinary succinate. Thus the fully-deuterated enzyme system was somehow better fitted to work on deuterated substrate, which of course is what it had been dealing with \textit{in vivo}. Even when the assay was done in $\text{H}_2\text{O}$ this remained qualitatively true, though there was then an isotope effect $k_H/k_D = 1.1$ or 1.2 depending on the pH of the assay. Since the results were similar whether $\text{D}_2\text{O}$ or $\text{H}_2\text{O}$ was the solvent in the assay, differences in hydrogen bonding of enzyme to substrate \textit{in vitro} cannot explain the main finding of Rittenberg and Borek.

Similarly, the non-exchangeable deuterons lowered the "melting" temperature of the protein phycocyanin isolated from fully-deuterated algae,\textsuperscript{3} regardless of whether the exchangeable positions were deuterated or protiated.

However, it is also clear that hydrogen bonds, especially as they contribute to macromolecules' structures, may very well be important in biological effects of deuterium. They are largely responsible for holding together at least the $\alpha$-helical parts of proteins' secondary structures,\textsuperscript{41} and slight changes have been measured in hydrogen bond lengths when deuterium is substituted for protium.\textsuperscript{16} Ribonuclease molecules are largely helical in aqueous solution at room temperature and unfold to a random coil when heated; in the
all-protiated enzyme, in H₂O, the mid-point of this transition is at 62°, but if the solvent is D₂O, at 66° (ref. 59).

As for nucleic acids in D₂O, it was to be expected⁵⁰ that even if there were only a very small isotope effect on the breaking of each hydrogen-bonded base pair the unravelling of double-stranded DNA or other complementary polynucleotide might be critically affected by a cooperative deuterium isotope effect. It therefore was perhaps a surprise that several investigations (e.g. refs. 11 and 42) of the thermal denaturing ("melting") of double-stranded DNA in D₂O and H₂O failed to show any difference in the mid-point, width or total hypochromicity. Similar negative results were found⁴² on three tRNAs and tobacco mosaic virus RNA. These were essentially thermodynamic measurements, whereas kinetics might be more relevant to the biological effects of D₂O. A brief note added in proof⁴² stated that there is no kinetic effect of D₂O on the renaturation of E. coli DNA. Recently the apparently-settled thermodynamic questions have been re-opened by the work of Lewin,³⁷ who finds that, at lower ionic strengths than had previously been used, D₂O does indeed change the melting point, raising it 10-15° (calf thymus DNA) or even 25-35° (salmon sperm DNA), in fair agreement with the maximum possible changes computed from a reasonable model of water bridges in DNA.

It has been suggested on theoretical grounds (e.g. ref. 39) that deuterium in place of protium in the hydrogen bonds of nucleic acids might affect their functioning, either the accuracy of copying in replication of DNA and in transcription, or perhaps the recognition
C. Aim of this Study

My experiments have tried to identify, from among the various possible molecular changes, some of those which actually do occur in cells growing in D₂O.

D. Choice of an Organism

The need was for an organism which showed biological effects of deuterium, but not so strongly as to kill it. It was already known that E. coli can grow, slowly, in D₂O. E. coli is the best-understood organism at the molecular level, particularly in respect of syntheses of macromolecules. Among its many advantages for biochemical work, those most relevant here are: (i) it can grow in simple, fully-defined media, (ii) it is far easier to work with and interpret than typical multicellular organisms, (iii) it grows at convenient rates, generation times in common media falling in the range 0.3 - 3 hr, and (iv) thousands of mutants have been isolated.

E. Control of Proteins' Synthesis

Many of my experiments were on the synthesis of one macromolecule, the enzyme β-galactosidase, systematically named β-D-galactoside galactohydrolase (Enzyme Commission 3.2.1.23), and abbreviated here to BG. Its function is believed to be the hydrolysis of lactose to galactose and glucose, but it can also catalyze many other trans-galactosidations. Since the theory of Jacob and Monod has largely determined our thinking on control of BG synthesis, let us briefly review its relevant parts. Mapped mutations of BG define the
structural gene of BG, called the $z$ gene, which specifies the structure of the BG molecule. The rate of expression of the $z$ gene is variable according to need: the wild type *E. coli* makes almost no BG (only a few molecules per cell) until presented with lactose, whereupon within a few minutes the rate of synthesis of BG rises several hundredfold. This induction can be even more effectively performed by some analogues of lactose. $\beta$-l-thio-D-galactosides such as isopropylthiogalactoside (IPTG) cause the rate of synthesis of BG to rise a thousandfold after a lag of 3 min in a culture growing at 37º. Whereas lactose is of course metabolized by the induced BG, it is believed that thiogalactosides are not metabolized. They are therefore termed gratuitous inducers. BG synthesis is under genetic control, investigated by selecting mutants which have lost control of BG synthesis so that even in the absence of an inducer BG is made at maximal or at least a very high rate. These are called constitutive mutants, and map in two genes, $i$ and $o$. The $o$ (operator) gene is very close to the $z$ gene but the $i$ gene is some little distance away. Suitable mating experiments can introduce an $i^+$ allele into an $i^-$ cell. The resulting partial diploid is inducible, not constitutive. (By contrast, the other type of constitutive, $o^c$, is unaffected in such an experiment.) This was interpreted to mean that the $i^+$ allele produces a diffusible molecule which acts to shut off the synthesis of BG, and was therefore called the repressor. By definition, the repressor's site of action is called the operator. It has been commonly assumed that the operator gene is the operator, but in fact the question of what the
operator is remained open for years. For example, in 1965 Watson rightly insisted (Ref. 65, p. 397) that it might be either the synthesis or the functioning of mRNA which is inhibited by the repressor. There even appeared in 1968 a whole book which was largely devoted to showing that we did not know where the rate of protein synthesis is controlled. However, the repressor of BG synthesis has now been extensively purified and its interaction with DNA studied. It is a tetrameric protein, MW 4 x 40,000, of uninteresting amino acid composition; is acidic, in contrast to the histones; and contains very little or no nucleic acid. It binds to DNA containing the wild-type o allele, but not to DNA from a 20%-constitutive o mutant. This experiment, though suggestive that the operator is DNA, still left the possibility that the repressor might act in vivo on the mRNA, which is presumably a transcript of at least one strand of the DNA. That hypothesis was weakened by failures to observe binding of the repressor to the best model of mRNA available, namely single-stranded (denatured) DNA. It should be pointed out here that perhaps the repressor binds in vivo to a hybrid of one strand of the DNA with mRNA.

On the other hand, there is no doubt that in some cases protein synthesis is regulated at translation. For example, enucleated cells of different species of the unicellular alga Acetabularia grow and differentiate in their normal, species-specific way. Thus it seems that there is no one general answer to the question of where protein synthesis is regulated. Why, after all, should there be? It is even
possible that a gene's expression might be regulated at both transcription and translation.\textsuperscript{61}

Let us return to the control of BG synthesis. Control mutants other than the constitutives $i^-$ and $o^c$ are known. Willson\textsuperscript{68} discovered the $i^8$ mutant, which produces an active repressor but which can be induced only by enormous concentrations of IPTG, and only to a small extent at that; and which is dominant to wild-type. These properties were predicted by the theory for a mutant whose repressor binds the operator normally but the inducer only weakly.

The $i$ and $o$ genes also control the expression of two structural genes other than $z$, namely its neighbors the $y$ and $ac$ genes. These code respectively for the galactoside pump (almost always called a permease, though this name implies that it is an enzyme when there is no good evidence for this), and for a protein of unknown function. Since nearly all early $y^-$ mutants were also found to be $ac^-$, it was at first thought that the ac gene coded for part of the galactoside pump, but now mutants are known of both $y$ and $ac$ singly.\textsuperscript{43} The protein encoded by the $y$ gene has been purified,\textsuperscript{15} though how it works to concentrate galactosides from the medium into the cell by two orders of magnitude remains largely mysterious. The only enzymatic function known for the ac gene's product is as a thiogalactoside transacetylase, which is presumably not its role in life because thiogalactosides have not been found in nature, and in any case $ac^+$ strains are unable to metabolize (artificial) thiogalactosides.\textsuperscript{29} In the wild-type, induction of the $z$, $y$ and $ac$ genes is co-ordinate; and they map very close together (in that order). Being controlled
by the same (i and o) genes, and encoding lactose-metabolizing enzymes, they are said to be part of the lac operon. There is some confusion over the definition of the term operon. As originally used it included the o gene (Ref. 29, p. 344), but later some have defined it in a different way which might exclude the o gene, e.g. (Ref. 65, p. 399): "The collections of adjacent nucleotides that code for single mRNA molecules and that are under the control of a single repressor, are called operons." This definition might exclude the o gene from the operon—we do not yet know whether the o gene is transcribed (though there is good evidence that it is not translated). Watson's definition also suffers from speculatively generalizing about the number of species of mRNA transcribed from each set of adjacent, co-ordinately expressed genes, a matter on which few if any facts are known.

Figure 1 (Ref. 65, p. 400) shows the theory of Jacob and Monod.

It was to be expected in a general way that we might learn something new about control of protein synthesis by studying it in D₂O. When the project began, no repressor had been purified, and there was even room for guessing whether repressors are protein and/or nucleic acid. There was accordingly very scanty information about the nature and strength of repressor-inducer and repressor-operator interactions. Since substitution of D for H in hydrogen bonds may change their strength, we thought it possible that we might find out something about such matters. On the whole, this hope has been frustrated, but late in the work an unexpected and apparently unique effect of D₂O on control did emerge. Two
Figure 1. The hypothesis currently favored for how the interaction of repressor, corepressor, and operator controls the synthesis of E. coli proteins β-galactosidase and β-galactoside permease. In this and in subsequent illustrations we show repressors combining with DNA. This point has not yet been proved, and the alternative hypothesis exists that repressors act by combining with mRNA, thereby preventing its attachment to ribosomes, and so allowing its rapid enzymatic breakdown to nucleotides. It is important to note that, under both sets of hypotheses, a specific repressor decreases the amount of a specific mRNA molecule.
and two constitutive strains of E. coli, after many generations in D₂O-acetate, showed thousandfold losses of ability to produce BG activity. The defect persisted after re-adaptation to H₂O-acetate, and was therefore thought to be a mutation z⁺ → z⁻. However, addition of glycerol restored inducibility, ruling out that hypothesis. The first question which then occurred was whether the lack of BG activity in cells growing on D₂O-acetate is due to failure to produce the enzyme, or to production of an inactive form of the enzyme. The latter result was found by gel electrophoresis of the soluble proteins. Full investigation of this strange effect will be a considerable project in itself, but should reveal new aspects of the control of gene expression.
II. METHODS

A. Abbreviations

BG  β-galactosidase
ONPG  o-nitrophenyl-β-D-galactoside
IPTG  isopropyl-1-thio-β-D-galactoside
tris  tris (hydroxymethylamino) aminomethane
TCA  trichloroacetic acid
MGT  mean generation time (mass doubling time of a culture)
P  differential rate of BG synthesis,
   \[ \frac{\partial (\text{BG activity})}{\partial \text{OD}_{650}} \n\]
trRNA  transfer RNA
mRNA  messenger RNA
Km  Michaelis constant (substrate concentration at which velocity is half maximal)
C  curie \( (2.22 \times 10^{12} \text{ dpm}) \)
dpm  disintegrations per minute
### B. Strains of E. coli Used

All strains were stated by their suppliers to be descended from strain K12.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Origin</th>
</tr>
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<tbody>
<tr>
<td>300U</td>
<td>$i^+ o^+ z^+ y^-$</td>
<td>J. Monod</td>
</tr>
<tr>
<td>230U</td>
<td>$i^- o^+ z^+ y^-$</td>
<td>J. Monod</td>
</tr>
<tr>
<td>2000 $o^c$</td>
<td>$i^o z^y$</td>
<td>C. Willson</td>
</tr>
<tr>
<td>$o^c_{67}$</td>
<td>(i and o deleted) $z^y^+$</td>
<td>E. Steers $^60$</td>
</tr>
<tr>
<td>Cavalli</td>
<td>$i^+ o^+ z^+ y^+$</td>
<td>A. Simmons</td>
</tr>
<tr>
<td>E 203</td>
<td>2 or 3 inducible lac operons.</td>
<td>A. Novick $^27$</td>
</tr>
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</table>

Since most of these strains had been cultured for many months (many hundreds of generations) without re-isolation, there was doubt about their purity. They were therefore re-isolated where possible. A technique which was often useful was growth on plates containing EMB-lac (eosin-methylenblue-lactose), $^45$ according to a recipe from Clyde Willson (personal communication): bacto-tryptone, 5 g; yeast extract, 2.5 g; NaCl, 5 g; agar, 11 g; lactose, 10 g; eosin Y, 0.2 g; leucomethylenblue, 0.032 g; water to 500 ml; autoclave. On this variant of EMB-lac, a colony which has not fermented lactose is light pink, like the plate itself (though a central papilla of dark blue often develops some days after the colony has grown large); but
a colony which has fermented lactose is very dark blue from the
time it is first visible. Examples of mutations which give rise
to pink colonies are \( z^- \), \( y^- \), and \( i^8 \).

Another useful technique selected BG constitutives. Suit-
ably diluted cultures plated on glycerol-minimal agar gave visible
separate colonies. The plates having then been inverted for 1-2
min over filter paper soaked with toluene, were irrigated with a
minimum volume of ONPG solution. Constitutive colonies turned
yellow within half a minute, whereas others took at least several
minutes. Another sample of a constitutive colony was then taken
from a replica plate made before the toluene treatment.

The re-isolations were done as follows.

300U: A light pink colony from EMB-lac was confirmed to have
a very low induced and a thousandfold-higher IPTG-induced \( P \) value.
It was therefore taken to be \( i^{+0}z^+y^- \).

230U: EMB-lac plates of this strain revealed a considerable
sprinkling of blue colonies, presumably \( y^+ \) revertants. A pink
colony had a very high \( P \) value, with or without inducer. It was
therefore taken to be \( i^{-0}z^+y^- \).

2000 \( o_C \): A constitutive colony from a glycerol-agar plate was
confirmed to be a partial constitutive, \( P = 3 \times 10^2 \), superinducible
by 0.5 mM IPTG to \( 3 \times 10^3 \).

\( o_C \): No simple tests nor selective procedures were available
for the genotype which this strain was stated to have; but since
the lac mutations in it were said to be deletions, which of course do not revert, it was thought sufficient simply to select a constitutive, which was assumed to be (i&d deleted) z⁺.

C. Media and Growth Conditions

Cells were grown at 37 ± 0.7° in M63, a liquid minimal medium, containing 2.7 g/l acetate, or the same concentration of carbon in the form of the indicated carbon source. When required, amino acids were present at 50 mg/l and thymine at 2 mg/l.

Cultures were maintained at 37° in liquid media subcultured every three days in H₂O or about every two weeks in D₂O, one drop being transferred to 10-50 ml so that one subculture corresponded to 8-10 generations of growth.

Fully-deuterated media were made as follows: The solid constituents and the acetic acid (d₄) were dissolved in the minimum volume of 99.8% D₂O (from AEC, Savannah River, Ga.; sometimes via ICN Co.) and the pD adjusted with solid KOH and NaOH. Since the formula for M63 ostensibly lacks sodium (i.e. depends on impurities to provide it), I thought it better to provide an excess of sodium rather than risking fluctuations of sodium concentration between batches of medium. The dissociation constants of H₂O and D₂O being different (Table I), it is not possible to make D₂O media with the pD and the pOD both to the corresponding values in H₂O. It was rather arbitrarily decided to make the pD equal to the pH of all-H M63. (A pH meter of which the electrodes have been soaked in D₂O for hours
reads \textsuperscript{19} \textit{pD} = 0.4.) The solution was freeze-dried after allowing ample time (hours) for exchange of protium on N, O, and S. The residue was dissolved in the minimum volume of 99.8\% D\textsubscript{2}O, freeze-dried again, dissolved in the final volume of 99.8\% D\textsubscript{2}O, and assayed for protium as described in Appendix I. For each batch of all-D medium the mole ratio D/H even after use was at least 200/1. Tritium content, assayed by liquid scintillation counting, was below 0.9 nC/ml for most of the work, and below 90 nC/ml for the rest.

Acetate (d\textsubscript{3}) was chosen as carbon source because the relatively slow growth rates on it should minimize catabolite repression; also it happens to be by far the cheapest fully-deuterated carbon source for \textit{E. coli}.

Carbon sources other than acetate, and supplements for auxotrophs, were enriched in deuterium at the non-exchangeable positions.

The only medium which was fully deuterated (including the hydrogen of the substrate) was the D\textsubscript{2}O-acetate (d\textsubscript{3}) medium described above. Since it was used for most experiments, it will be referred to conveniently as \textit{all-D}. It will also be a convenient shorthand to use the term \textit{all-D} for cells which have grown in this medium. The corresponding medium made from H\textsubscript{2}O and ordinary protiated acetate, or cells from this medium, will be called \textit{all-H}.

Measurements were made on 10-50 ml cultures which had been growing exponentially for at least one generation, usually two.

Because D\textsubscript{2}O can readily exchange with and thus become diluted by H\textsubscript{2}O vapor in the air, it must be treated as hygroscopic. Rubber
stoppers were therefore used on growth flasks instead of the more usual cotton plugs, and sterilization was by means of Millipore filters (type HAWP, 0.45 μ pores; Millipore Corp., Bedford, Mass.) instead of autoclaving. The filters were mounted in their holders, with a disc of thick pre-filter placed on the filter to prevent buckling. A narrow-bore inverted U-tube with cotton plug was fitted through an extra hole in the filter-holder's stopper to allow escape of displaced air during the filtering. The mounted filter was wrapped in foil, autoclaved (20 min at 121°), and dried. The all-D medium was forced through the filter into a sterile, dry 250-ml Erlenmeyer flask. The filter was then quickly transferred, with brief flaming, to another such flask, and replaced by a sterile, dry rubber stopper. The flasks of filtered medium were kept at room temperature for at least two weeks to check sterility.

Several attempts to adapt cultures by a sudden shift from H_2O to 99% D_2O failed, so a serial training was adopted: 0→20→40→80→90→99% D_2O. One drop of resting culture was transferred at each step. This method usually worked, but in a few cases the cells failed to grow at the last step. An even more gradual series at high D_2O concentrations was therefore arranged by removing at each step half of the culture and replacing it with the same volume of >99.5% D_2O medium. Growth was allowed to proceed for about one generation at each step, and was conveniently followed aseptically in a flask with a sidearm into which the culture could be poured to read its turbidity in a spectrophotometer. The series thus produced was nominally 0→50→75→88→94→97→98→99% D_2O. During this adaptation,
lactose was the sole carbon source, to select for ability to make BG. One drop of the 99% D$_2$O culture was transferred to all-D medium, whereupon a lag of several days usually occurred, and in some cases the culture even failed to grow after many weeks. A few experiments led to the tentative notion that it helped to use protiated acetate at this stage, and then transfer to all-D after several generations.

D. Measurement of Growth

Previous work in this laboratory$^{47,51}$ has shown that, in H$_2$O under a wide range of conditions of growth, the optical density of *E. coli* cultures at 650 nm (a wavelength at which they scatter much more than they absorb) is proportional to the concentration of protein in the cultures: over the range OD$_{650}$ = 0.1 to 1.8, µg protein/ml = OD$_{650}$ x 225. For the comparison of all-H and all-D cultures, two questions arose: is the OD linear with total protein in the all-D cultures, and is the constant of proportionality different from the value for all-H cultures? The first question was answered by measuring the incorporation of L-phenylalanine-U-$^{14}$C by Cavalli which was in its tenth subculture in all-D. From OD .07 to 0.4 the incorporation was linear. The second question was answered by comparing the total yield of amino acids from acid hydrolysis of all-D and all-H Cavalli from which the free amino acids had been extracted. This measurement (see Results) showed that the protein content per OD$_{650}$ was double in the all-D cells. What then does OD$_{650}$ mean in a deuterated culture? It was shown to measure wet weight of cells
per unit volume of culture by a simple method: millipore-filtering exponential cultures to and from equal volumes of all-H and all-D media always gave the same OD$_{650}$ after filtering as before, within 5%. In our hands this method has consistently given at least 95% efficient transfer in an all-H→all-H shift, so that the conservation of OD$_{650}$ in all-H→all-D and all-D→all-H shifts means that OD$_{650}$ measures wet cell weight.

OD$_{650}$ was measured by sampling about one ml of culture into a cuvette of path length 1.00 cm and reading the optical density at 650 nm in a Beckman DK2, Beckman DU / Gilford 220-209, or Cary 14 spectrophotometer. Since the fraction of unscattered light is what is measured, the apparent OD depends (as it does not in the case of actual absorption) on the distance of the detector from the cuvette, so that the three instruments differ by about 10%. It is therefore particularly necessary to do a given experiment on one instrument. However, the growth curves from all three for a given experiment are parallel.

E. Induction and Assay of β-Galactosidase

Isopropyl-1-thio-β-D-galactoside (IPTG), not enriched in deuterium but dissolved in D$_2$O when used to induce an all-D culture, was added as a 50 mM solution to give final concentration in the cultures of 0.5 mM (except where other concentrations are specified). Samples of culture were taken by short Pasteur pipettes on adjustable syringe controls, into an equal volume of chloramphenicol (1 mg/ml) to stop protein synthesis. Usually 0.2 ml samples were used.
The assay depends on the hydrolysis of the colorless substrate \( \text{o-nitrophenyl-\(\beta\)-D-galactoside (ONPG), which is hydrolyzed by BG to give yellow o-nitrophenol. In order to make the enzyme more accessible to the substrate, and in particular to abolish the effect of the galactoside pump, the cells are shaken with toluene. The reaction is conveniently stopped by adding base to denature the BG. Since low activities often require assay times of hours, during which the non-enzymatic hydrolysis of ONPG is not negligible, a blank is always run and used as the reference solution in the double-beam spectrophotometer while measuring the yellow color produced by the BG.

Most of the assays used the following modification of Kepes' method. \(^{33}\) After shaking for a few seconds at the time of sampling into chloramphenicol, the samples, sometimes kept for a day or two at 4\(^\circ\), were vigorously vortexed at room temperature with a drop (ca. 20 \(\mu l\)) of toluene for 5-10 sec. They were warmed to 37\(^\circ\) and the assay was started by adding 0.80 ml of 3.3 mM ONPG in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.125 M NaCl. When an easily visible yellow had developed, the reaction was stopped by adding 0.2 ml of 1.5 M \(\text{Na}_2\text{CO}_3\). Cells and debris were centrifuged for about one hour at 5,000 \(x\) \(g\) and the \(\text{OD}_{420}\) of the nitrophenolate was read in a Cary 14 spectrophotometer. One unit of enzyme activity is defined as the hydrolysis (in excess of the blank) of 1 nmole of substrate per minute under these conditions. Sometimes it was convenient to increase the assay time by doing it at a lower temperature. The rates were then corrected to 37\(^\circ\) using the temperature coefficient \(^{51}\) \(Q_{10} = 1.68\).
When the greatest possible precision was needed, the following improved assay method, developed in this laboratory, was used. Samples (30-100 μl) were added to clean weighed 10 ml stoppered vials containing 0.2 ml of chloramphenical solution, and the vials were re-weighed to correct for sampling error. One very small drop (ca. 10 μl) of toluene was added to each sample and the vials were shaken at 37° for 1/2-1 hr. The stoppers were then removed and the toluene allowed to evaporate for 1/2 hr. The assay was started as before, and stopped by mixing with 0.80 ml of 0.75 M Na₂CO₃. Centrifugation of solids was aided by adding about 0.1 g of solid Ba₂CO₃ and vortexing, after which only 1 min at top speed in a clinical bench centrifuge removed turbidity.

The usual way to plot production of BG activity by a growing culture is to show BG activity per ml against turbidity of the culture. Since both OD₆₅₀ and BG concentration usually increase exponentially, this plot usually gives straight lines. The slope of such a graph measures the rate of synthesis of BG activity with respect to cell wet weight, the so-called differential rate of BG synthesis:²⁵

\[
\frac{\partial (\text{BG activity/ml})}{\partial (\text{OD}_{650})} = P
\]

(The units of P are thus nmoles of ONPG hydrolyzed by 1 ml of culture, per unit of OD₆₅₀ of that culture.)
F. Shifts to and from D₂O Media

To shift cells between different media with minimal disturbance, the simplest and sometimes best way is to place a very small volume of one culture into a relatively large volume of the other medium which has been stirring at the same temperature. This method has important drawbacks for some applications, though. Firstly, if the cells have been induced in 0.1 mM IPTG, they would have to be diluted about one thousandfold to ensure that induction stopped; and the resultant density of culture would be too low to measure growth by turbidity. Secondly, since I found indications (Appendix II) that even 1% H₂O in 99% D₂O affects BG synthesis, it became important to ensure that in shifts from H₂O to D₂O as little medium as possible was carried over.

The following procedure was therefore used. Everything was done in a 37° room. Cells were suction-filtered on a Millipore filter (type RAWP, 47 mm dia., 1.2 μ pores) and washed with 5 x 5 ml of the kind of medium in which they were to be re-suspended, the cells being sucked just barely dry each time. It was found that at least 20 ml of a culture as dense as OD₆₅₀ = 0.4 could be filtered without significant clogging of the filter. The filter was quickly transferred to the new medium which had been stirring in the 37° room for at least an hour. Rapid stirring for about one minute gave at least 70% re-suspension, but a few minutes was necessary for maximum yield (ca. 95%).
G. Radiochemical Assays of Macromolecules

Cumulative labeling. To measure total DNA and protein, cultures were grown from a negligible inoculum (i.e., measurements did not begin until about seven generations of growth after inoculation) in a medium containing L-methionine-Me-\(^{14}\)C (10 mC/g) and thymine-Me-\(^{3}\)H (1300 mC/g). The solutions of radioactive compounds were evaporated to dryness before being dissolved in media. Fifty ml of the resulting all-D supplemented medium was evaporated to dryness and re-dissolved in 50 ml of H\(_2\)O. This method ensured that the specific activity of each radioactive compound would be exactly the same in the two media.

Samples were taken during at least one generation, 0.20 ml being mixed with 0.20 ml of ice-cold trichloroacetic acid.\(^{47}\) After 1-3 hr at 0\(^{\circ}\) the precipitate was collected on a pre-wetted Millipore filter (0.45 \(\mu\) pores) and washed with 3 x 1 ml of ice-cold 5% TCA followed by 10 x 1 ml of ice-cold water. Having been sucked roughly dry, the whole filter was dissolved in scintillation solution\(^{34}\) which was then agitated for a few minutes and gelled with "Cab-o-Sil" silica gel powder. A Packard counter with external standard, and a computer program correcting for both \(^{3}\)H counts in channel 2 and \(^{14}\)C counts in channel 1, gave the dpm of each radioisotope.

Pulse labeling. To measure rates of synthesis of DNA, RNA and protein, 0.5 ml samples of growing, non-radioactive cultures were added to 10 \(\mu\)l mixtures of radioactive uracil and phenylalanine, or
thymine and phenylalanine. After incubation at 37° with shaking, 
0.50 ml of 10% TCA was added and treatment continued as above, ex-
cept that the wash solutions were saturated with carriers for the 
radioactive precursors. When the culture from which the samples 
were being taken was all-D, the solvent for the radioactive com-
pounds was D₂O. The final amounts present during the pulses were: 
L-phenylalanine-U-¹⁴C, 0.25 µ Ci at 1 C/mole; uracil-5-³H, 2.0 µ C at 
2600 C/mole; thymine-Me-³H, 2.5 µ C at 60 C/mole.

H. Amino Acid Analysis

Exponential growth was followed for two generations and then 
the cells were harvested by centrifuging about 1.5 generations 
short of resting phase, 20 ml of OD₆₅₀ 0.400 (all-H culture) and 
20 ml of OD₆₅₀ 0.200 (all-D culture). The pellets were suspended 
in 75% ethanol at 45° for 30 min with occasional stirring. This 
treatment extracts free amino acids but very little protein.⁵₆

The two batches of cells were re-centrifuged and re-suspended, each 
in 1.0 ml of 6 M HCl, frozen at -190° in thick-walled, 10 ml tubes, 
and evacuated with an oil pump. The samples were then sealed and 
heated side by side at 110 ± 5° for 8 hours. HCl was removed at 
40-50° on a rotary evaporator with an oil pump. The residues were 
re-dissolved in H₂O, again dried by the same method, and taken up 
in 2.00 ml each of citrate buffer, pH 2.2, of which 0.20 ml of the 
protiated sample and 0.10 ml of the deuterated sample were applied 
to each column of a Beckman 120C amino acid analyzer. Flow rates
were 70 ml/hr for the eluting buffer and 35 ml/hr for the ninhydrin solution. Columns were thermostatted at 55°. The column for neutral and acidic amino acids (Beckman UR 30 resin) was eluted by 0.2 M sodium citrate, pH 3.25, until valine had been eluted, and thereafter by 0.35 M sodium citrate, pH 4.00. The column for basic amino acids (Beckman PA 35 resin) was eluted by pH 5.25 buffer. The absorption at 570 nm of the ninhydrin color of the effluent was integrated by an Infotronics CRS-110A integrator. This instrument avoids inaccuracies due to long-term baseline drift because it refrains from integrating until the rate of change of ninhydrin color exceeds a threshold value. Integration was calibrated by running on the same column a standard mixture containing 15.0 nmole of each amino acid. For different amino acids the ratio of standard to unknown peak areas varied from 7 to 0.1. Estimation of proline was by direct measurement of peak areas on the chart of time against OD_{440}.

I. Analytical Polyacrylamide-gel Electrophoresis of Proteins

First experiments were with the simplified method of Clarke, which gave 22-25 visible bands of protein but which is hard to scan quantitatively because the gels are in the form of rods. The results shown are from the commercial apparatus of the E-C Corp., Philadelphia, using a single-gel slab of 7.5% acrylamide and a tris-glycine buffer of pH 8.0. Exponential cells (20 ml of culture at OD_{650} = 0.4, or the equivalent) were centrifuged, washed and
kept at -20° until lysis. The best way to lyse E. coli cells is the lysozyme-detergent method of Godson,20 but two attempts to lyse deuterated cells this way failed. Therefore, the results shown are on cells disrupted by sonication. The pellet of washed cells was re-suspended in 0.5 ml of carbon-free M63 and sonicated for four 5-sec periods at full power of the Bronwill Biosonik, tuned to give a rushing sound as of a mighty wind, the sample being surrounded during sonication by an ice-water bath, and frozen on dry ice between bursts of the sonicator. After one hour at 25,000 x g, the supernatant was further concentrated by soaking in it for 24-30 hr a pellet of polyacrylamide-gel stated by the manufacturer (E-C Corp., Philadelphia) to absorb 310 ± 5 µl of water, but no proteins.

A single gel slab can run 16 samples simultaneously, but it was found necessary to run two or three dosages of each (usually 10, 20, and 30 µl) in order to get the best compromise between sensitivity and resolution. All the results shown are from the same gel slab. After staining for protein with the dye amidoschwartz 10B, and electrophoretic removal of unbound stain, the bands were scanned with a Goerz "Servogor" recording densitometer. The slit width was 3 mm, slit length 0.2 mm, and sensitivity about 50 mV.

Coomassie Brilliant Blue R250 (Colab Labs., Chicago Heights, Ill.), has been reported8 to give both increased sensitivity for minor bands and less blurring of heavy bands compared with amidoschartz, but we have found it inferior.
Michaelis constants. Ten ml of late-exponential culture was harvested on a Millipore filter and washed several times with acetate medium made from D$_2$O or H$_2$O, according to the solvent in which the $K_m$ was to be determined. The cells were re-suspended in 5 ml of the same medium, and broken in the French press. In a 1 cm cuvette, 0.20 ml samples of a 1:9 dilution of the broken cells were rapidly mixed with 0.80 ml of ONPG solution and the initial reaction rate measured as the rate of increase of $OD_{420}$ in a Cary 14 recording spectrophotometer. In drawing the lines on the Woolf plot a subjective weighting was made to allow for the fact that at higher 1/S values the rates are less certain.

Turnover numbers of pure BG. The simplified single-gel method of Clarke gave very pure BG so that the complicated three-gel method was not used. Purity was measured, on the all-H BG, as catalytic activity (under standard conditions) per equivalent $\mu$g of bovine serum albumin determined by the Lowry method.

E203 was adapted on acetate by the most gradual sequence to all-D. A 500-ml all-D culture was induced with IPTG and grown for about seven generations. The all-H culture was from a tiny inoculum of this all-D culture. Cells were centrifuged in late exponential or early resting phase, re-suspended in 1/20 of the supernatant, and made .01 M in 2-mercaptoethanol, which tends to stabilize BG. The suspension was surrounded by an ice bath and sonicated for six
one-min bursts of full power on the Bronwill Biosonik, with two-min pauses for cooling. Dialysis of the sonicate, recommended for better electrophoretic separation, was found to be unnecessary. Attempts to form gels containing 2-mercaptoethanol failed. Cylindrical gels 6 x 75 mm on which about 200 μg of soluble protein had been separated for 2-4 hr at 3-5 mA per gel showed at least 17 bands stained by amidoschwartz. When extracts of uninduced and induced E203 were run and stained side by side in the same gel, the only visible difference was a very heavy band which had run about 1/5 as far as the salt front; this band was almost certainly BG. To detect it without fixing it to the gel and destroying its activity, it was necessary to roll the gel on a filter paper wet with ONPG solution. Whereas extracts from all-H cells gave a sharp yellow band on the gel, those from all-D cells repeatedly gave only a streaked yellow band covering the uppermost 1/5 of the gel. Various loadings and times of electrophoresis did not improve this. The yellow zone had, however, a sharp front. The two or three mm of gel immediately behind this front was cut out and held on the top of a fresh gel with a cotton wad. Electrophoresis of this (the nitrophenolate acting as an indicator of the salt front) gave a single sharp band of BG activity. BG was extracted from the gels by grinding the excised BG-active gel band in a tissue homogenizer with 2-3 vol. of the buffer which was to be used in the assay, and standing overnight. Centrifuging for 10 min at 10,000 x g removed the gel. The supernatant, despite containing 2-mercaptoethanol (.1 M) and being kept frozen, lost nearly all of its BG activity within a few weeks.
The buffer and concentration of ONPG were the same as in the standard assay of toluenized cells (section E, above). The temperature was 21°. Protein concentrations of BG solutions, on duplicate samples, was by the Lowry method as modified for increased sensitivity. The standard curve was made from a range of dilutions of a standard solution of bovine serum albumin (Armour Meat Co., Chicago: vial of BSA solution, 10 mg/ml). The BG samples measured by the Lowry method were in the range 50-150 μg, which was well within the range of the standard curve. BG concentrations in the BG kinetic assays were nominally in the range around 1 μg/ml (and were known accurately from the concentration of the stock BG solution and the dilution factor for the kinetic assay).
A. Adaptation to and from $D_2O$

Although the main purpose of the adaptation procedures was of course merely to get fully-deuterated cultures, some interesting results emerged from them.

Variability in adaptation experiments was significant. No strain showed any consistently superior adaptability compared with other strains; the variability was as great between different adaptation attempts with the same strain as it was in comparisons between strains. A few cultures did grow within hours after the direct transfer from all-$H$ to all-$D$, but most did not grow within weeks.

In one experiment when cells which had suffered a lag of several hours after transfer from all-$H$ were growing slowly in all-$D$ with a MGT of 20 hr, microscopy showed that most of them were bigger than normal, and about one in twenty was a monster, eight or ten times longer than normal. A pulse-height analyzing Coulter counter gave the same qualitative result (Figure 5), but unfortunately could not be calibrated for quantitation of this effect. After adaptation to all-$D$, the cells looked normal under the microscope. This shows that the inhibition of cell division with respect to growth was no longer spectacular after adaptation to all-$D$, but light microscopy cannot distinguish a scaling-up by a factor of two in volume, because
linear dimensions will in that case be greater by a factor of only about 1.3, which is too small to measure reliably (Dr. Tamotsu Kanazawa, personal communication).

Even when serial transfer was resorted to, some cultures did not grow after the transfer from 90% D$_2$O to 99% D$_2$O. This agrees with previous results of other workers who have found that algae (for example) can readily adapt to moderate concentrations of D$_2$O (about 60%) but are very hard to grow at concentrations above 90%.

Quantitative investigation of the dependence of Cavalli's growth rate on percentage of D$_2$O (with acetate as carbon source) revealed that growth is prompt and fast in 0% D$_2$O, 20% D$_2$O, and 40% D$_2$O, after inoculation from either all-H or all-D (Figures 2 and 3). One aspect of this result is in disagreement with the previous work of Borek and Rittenberg, who found a lag of several hours (as measured by turbidity and by cell counts) when a K12 strain of E. coli was shifted from D$_2$O into H$_2$O. My result has been obtained so often on several K12 strains that we must look into the possible reasons for the disagreement. Although Borek and Rittenberg did not describe the preparation of their D$_2$O medium, it is unlikely to have been more fully deuterated than mine, so that we may neglect the possibility that their experiment entailed a bigger shift of isotopic abundance. More likely to be significant is the fact that the carbon source in their experiments was glucose (not enriched in deuterium). It is known that growth on glucose leads to a complement of enzymes somewhat different from that produced by growth on acetate. Therefore it is possible that in some way the use of glucose instead of
Figure 2. Growth of Cavalli shifted at time zero from all-H into various percentages of D$_2$O: ○, 0%; ●, 20%; ▲, 40%, △, 96%; and □, 99%. Carbon source throughout was acetate.
Figure 3. Growth of Cavalli shifted at time zero from all-D into various percentages of D₂O, as in Figure 2.
acetate was the critical difference. Since Borek and Rittenberg did a control H₂O→H₂O shift, which produced no lag, it seems clear that their finding was not an artifact. My results, on the other hand, have shown clearly that cells adapted to D₂O need not always lose ability to grow in H₂O. I even found several times (e.g., Figures 22 and 23) that not only did growth resume within a few minutes after an all-D→all-H shift, but also the rate was faster than the definitive rate in all-H, MGT values being 1.3–1.8 hr compared with the usual 2.0–2.5 hr. Unusually fast growth in all-H after a shift from all-D can be rationalized by an extension of Orgel's theory, as follows. If the fully-adapted all-D cell has more molecules of many enzymes, and if turnover numbers are nearly restored to normal by rapid exchange of H for D on S, N and O, the cell will have greater catalytic power than it has ever had (for many reactions, at least), so that for a time which might be estimated at one or two generations there might well be extra-fast growth. This model also offers a way to account for the fact (Table IV) that as long as 1.5 generations (but not 2.0 generations) after this shift from all-D to all-H the culture can still re-adapt to all-D much more readily than can a culture which has never been in D₂O. This fact appears puzzling at first, since after the 1.5 generations in all-H about 2/3 of the macromolecules and essentially all of the small molecules such as free amino acids are presumably fully-protiated, so that the cells are in isotopic composition much nearer all-H than all-D. However, the unusually large
numbers of enzyme molecules might easily persist that long, at least
to an extent sufficient that growth back in all-D would be helped.

B. Protein/DNA Ratios

To test Orgel's hypothesis that adaptation to D₂O consists of
de-repression of many enzymes, which would result in a higher pro-
tein/DNA ratio after adaptation, cumulative incorporation of L-
methionine-Me-¹⁴C and thymine-Me-³⁻H was measured in strain Cavalli,
which requires both methionine and thymine for growth. The all-D
culture had grown about 30 generations in all-D, and the all-H
culture about 20 generations since returning to all-H from all-D.
Figure 4 shows that the all-D culture incorporated 2.1 times more
methionine per gram of thymine than did the all-H culture; its
protein/DNA ratio was therefore 2.1 times higher.

Methionine was used because Cavalli cannot synthesize it, so
that the specific activity of methionine in the proteins cannot be
diluted by any endogenous methionine which, in a prototroph, might
continue to be made despite the large external supply of methionine.
However, the use of methyl-labeled methionine might seem unfortunate,
because this methyl group is used as a source of methyls in various
molecules other than proteins. For example, transfer RNA contains
thymine, the methyl of which originates in the S-methyl of methio-
nine. Quantitatively, though, Charles Prevost (unpublished results
from this laboratory) found that an undetectable activity (less than
0.1%) of the ¹⁴C incorporated into macromolecules from L-methionine-
Figure 4. Incorporation of thymine-$^3$H and L-methionine-$^{14}$C into TCA-precipitable material by exponential Cavalli, all-H (o) and all-D ($\Delta$). The ratio of slopes is 2.1.
Me\textsuperscript{14}C is not in methionine. For our present purposes, then, we may take it that proteins were the only macromolecules labeled significantly with \textsuperscript{14}C.

The result in Figure 4 might be at least partly due to an increased fraction of methionine in the proteins of the all-D cells. To test this hypothesis, and also as another general index of how different the all-D cells are, the amino acid composition of the protein in all-D and all-H Cavalli was measured.

C. Amino Acid Composition

The hydrolysis time of 8 hr was chosen because a kinetic study of release and degradation of amino acids from protein in 6 M HCl at 110° had shown\textsuperscript{56} that 8 hr is, on the one hand, long enough to release almost all amino acids completely, and on the other hand, short enough that, even when evacuation of the hydrolysis tube is omitted, degradation is not extensive. Figure 6 shows the chromatograms of the hydrolysates. Since only free amino acids and some lipids had been extracted from the cells before hydrolysis, the chromatograms seem remarkably simple even though the detection system responds rather selectively by detecting only ninhydrin-positive compounds. The comparison (Table II) between the two samples gains confidence from the fact that the total yield of amino acids eluted from the protiated mixture corresponded closely to the estimated weight of protein in the original sample of all-H culture (20 ml at OD\textsubscript{650} = 0.40 contains\textsuperscript{47} about 1.8 mg total protein). The typical degradation products of methionine, its
Table II. Amino acid composition of acid hydrolysates of protiated and deuterated *E. coli* strain 300U

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Retention time of peak (min)</th>
<th>Yield (μg)</th>
<th>Mole (%)&lt;sub&gt;u&lt;/sub&gt;</th>
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<td>D</td>
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<td>Glycine</td>
<td>86</td>
<td>87</td>
<td>6.54</td>
</tr>
<tr>
<td>Histidine</td>
<td>58</td>
<td>57</td>
<td>2.44</td>
</tr>
<tr>
<td>Isoleucine</td>
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<td>150</td>
<td>6.13</td>
</tr>
<tr>
<td>allo-Isoleucine&lt;sup&gt;q&lt;/sup&gt;</td>
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<td>141</td>
<td>0.44</td>
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<tr>
<td>Leucine</td>
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<td>156</td>
<td>10.98</td>
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<tr>
<td>Lysine</td>
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<tr>
<td>Methionine</td>
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<td>1.47</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>187</td>
<td>191</td>
<td>6.09</td>
</tr>
<tr>
<td>Proline&lt;sup&gt;r&lt;/sup&gt;</td>
<td>71</td>
<td>72</td>
<td>4.04</td>
</tr>
<tr>
<td>Serine</td>
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<td>54</td>
<td>4.83</td>
</tr>
<tr>
<td>Threonine</td>
<td>51</td>
<td>51</td>
<td>6.27</td>
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<tr>
<td>Tyrosine</td>
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<td>183</td>
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<tr>
<td>Valine</td>
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<td>94</td>
<td>7.50</td>
</tr>
<tr>
<td>A&lt;sup&gt;s&lt;/sup&gt;</td>
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<td>--</td>
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<tr>
<td>B&lt;sup&gt;t&lt;/sup&gt;</td>
<td>80</td>
<td>79</td>
<td>--</td>
</tr>
<tr>
<td>(Ammonia)</td>
<td>74</td>
<td>72</td>
<td></td>
</tr>
</tbody>
</table>

115 μg 67 μg
Footnotes to Table II

p  Arparagine and glutamine are completely converted to the corresponding amino acids aspartic and glutamic by the method of acid hydrolysis used.

q  Identification by retention time; color constant of isoleucine used.

r  Determined by peak area measured directly on chart of the 440 nm absorbance trace. (All other peaks were determined by digital integration of the 570 nm absorbance.)

s  This peak may be β-alanine, urea or taurine. Its retention time is significantly different from that of tryptophan. An average color constant was assumed.

t  Appears to be a doublet. May include methylamine. An average color constant assumed.

u  Since tryptophan and cysteine are not determined by this method, their mole % values have been taken as 1.7 and 1.04 respectively. It is not asserted that these values hold for deuterated cells; the purpose of the assumption is only to bring those mole % values which were determined to a total protein basis, as nearly as possible.
sulfoxide and sulfone, are known to elute near glutamic acid and appear to be absent from the chromatograms, as was expected from the relatively short time for which the samples were heated at 110° and from the thorough evacuation of the tubes before heating.

Although systematic errors in the amino acid analyzer are smaller than many of the apparent differences between the compositions of the deuterated and protiated cells, possible random errors make it best to conclude only that if there is any difference in the methionine contents it tends to re-enforce rather than diminish the difference of protein/DNA ratio indicated by the incorporation experiment (Figure 4).

Future investigators interested in properties of deuterated amino acids might investigate whether the apparent differences of retention time in Figure 6 are real.

The method used here is not comprehensive, in that it does not determine tryptophan or cysteine, and does not distinguish the amides asparagine and glutamine from aspartic acid and glutamic acid, respectively. Nevertheless it is clear that the amino acid composition of all-D protein is very similar to that of all-H protein. Such a finding is consistent with the observation (see below) that the quantitative profile of soluble proteins of all-H cells resembles that of all-D cells. Thus, there is no evidence from my experiments that all-D cells make a notably disproportionate amount of any one protein. There are minor differences, however; these will be discussed below (Figure 19).
From Table II we can also get an estimate of the relative protein content per gram wet weight of the two cultures. Since the all-D hydrolysate originated in 20.0 ml of OD$_{650}$ = 0.200 culture, whereas the all-H hydrolysate was of 20.0 ml of OD$_{650}$ = 0.400 culture, and since the fraction chromatographed of the all-D culture was 0.100/2.00, whereas the fraction of the all-H hydrolysate chromatographed was 0.200/2.00, the relative protein content on a wet weight basis is (67/115 x 0.4/0.2 x 0.2/0.1) = 2.3. Therefore the higher protein/DNA ratio in the all-D cells is largely due to more protein rather than less DNA.

As would be expected from this result, the deuterated cells are bigger. Figure 5 shows the size distribution, measured on a simultaneous pulse-height analyzing Coulter counter which responds to cell volume (rather than, for example, cross-sectional area), but could not be calibrated with the standard polystyrene balls because for some unknown reason they did not give counts. This matter deserves more investigation. For example, one would like to know whether the fully-adapted cells change further in cell size distribution.

D. Kinetics of Catalysis of Deuterated β-Galactosidase

In comparing the production of BG by all-H and all-D cultures, it is convenient to use the catalytic activity as a measure of the concentration of BG in the culture. However, the assumption is questionable that the constant of proportionality between molarity of BG and BG activity is the same for all-H and all-D BG. That is, the turnover number under the standard assay conditions (in H$_2$)
Figure 5. Measurements by pulse-height analyzing Coulter counter of cell volume distributions in deuterated and protiated Cavalli: a) all-H cells, stationary phase; b) all-D cells, stationary phase, and c) all-D cells, exponential phase.
Figure 6. Ion-exchange chromatograms on an amino acid analyzer of ninhydrin-positive compounds in acid hydrolysates of 75% EtOH-extracted Cavalli. In each pair of traces the upper is from all-H cells, the lower from all-D. The upper pair of chromatograms is of acidic and neutral compounds, the lower pair basic compounds chromatographed on a different column. The dashed portions are at tenfold higher sensitivity.
might be different for all-D BG. The question is also interesting
in itself: it is conceivable that enzyme activity might be changed
by full substitution of deuterium for protium on the non-exchangeable
positions (bonded to carbon).

A preliminary study measured the Michaelis constant \( K_m \) of the
deuterated and the normal BG of strain \( o^c_{67} \). \( K_m \) is often\(^{14} \) a good
measure of binding of substrate to enzyme; it is one factor influencing
turnover number, though not the only one. The culture used as the
source of deuterated BG in this experiment was \( o^c_{67} \) which had been
adapted to and maintained on \( D_2O \) using lactose as sole carbon source
throughout, to preserve maximum ability to synthesize BG. The lac-
tose was not enriched in deuterium, but the BG should nevertheless
have been almost as highly deuterated as if it had been produced in
all-D, because inspection of the metabolic pathways by which lactose
is used shows that almost all of the protium bonded to carbon will
exchange with the \( D_2O \) (diluting the latter to a negligible extent)
in the reactions by which carbon is incorporated into amino acids
and then into protein. The protiated BG was from \( o^c_{67} \) re-adapted
from \( D_2O \)-lactose to \( H_2O \)-lactose. Figure 7 shows that there probably
is a significant solvent effect on the \( K_m \) of the protiated enzyme,
but allowing for the estimated experimental uncertainty there is
probably not a significant effect on \( K_m \) from deuteration at the
non-exchangeable positions. This experiment measured \( K_m \) values
under conditions approximating those \textit{in vivo}, but does not give
turnover numbers applying to the standard assay conditions. There-
fore it was necessary to measure the catalytic activity of a known
Figure 7. Woolf plots \(^{14}\) to determine \(K_m\) of BG.

- **o**, BG from all-D cells, in D\(_2\)O assay buffer \(K_m = 0.16\) mM;
- **△**, " " " " H\(_2\)O " " " " 0.176 mM;
- **o**, " " all-H " " " " 0.13 mM;
- **△**, " " " " D\(_2\)O " " " " 0.186 mM.
concentration of the two forms of the enzyme. For this purpose the enzyme must be very highly purified so that an analysis for protein, such as the Lowry method, will permit calculation of the molarity of BG. Whereas the Lowry assay is known to vary in sensitivity with amino acid composition of proteins, it seems reasonable to assume that the all-D BG will have the same amino acid composition as the all-H BG, and that the deuterons bonded to carbon will not significantly change the sensitivity of the Lowry assay.

The standard method for isolating BG from E. coli uses fractional precipitation with ammonium sulfate, followed by two steps of column chromatography. This procedure would be necessary for a yield of milligrams, but for the present purpose of measuring kinetics for smaller amounts suffice, so that the analytical method, namely polyacrylamide-gel electrophoresis, used to monitor purifications at the successive steps of the preparative method will yield enough.

To conserve D2O, it was particularly necessary to maximize the yield of BG per liter of culture by using the super-producing strain E203. Selected in a chemostat by growth on limiting lactose, this strain produces two or three times the normal fully-induced P, presumably owing to the presence of two or three lac operons per cell.

The principal question as to turnover number in the standard assay was answered as follows (Table III): the catalytic activity per mole of pure BG from all-H and all-D cells in the H2O buffer used for the standard BG assays, was the same within 25%. The
Table III. Turnover numbers at 21° of BG from fully-deuterated cells and from fully-protiated cells.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Turnover number (moles of ONPG/sec, for 1 µg of BG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protiated</td>
<td>5.0 x 10^{-9}</td>
</tr>
<tr>
<td>Deuterated</td>
<td>4.8 x 10^{-9}</td>
</tr>
<tr>
<td>Protiated (data of Cohn)</td>
<td>3.9 x 10^{-9}</td>
</tr>
</tbody>
</table>

actual activities measured were within 5%, but allowing also for other sources of error outside the kinetic measurement itself, it is estimated that the limits of uncertainty were ± 25%.

If the improvement on Cohn's previous turnover number is real, it could be due to the preserving effect of mercaptoethanol, or simply to the superior purification power of polyacrylamide-gel electrophoresis over the methods available to Cohn in his 1957 work.

It might be objected that perhaps these results do not apply to strains other than E203. It was recognized by those who selected E203 that not only super-production of BG but also super-efficient BG might be selected by growth on limiting lactose. They showed that the BG of E203 had the normal $K_m$ and thermal inactivation profile; and more significantly, that the concentration of BG in the cells, analyzed by ultracentrifugation, was proportional to the BG...
activity as the selection proceeded. The conclusion was confident that E203 produces more, not better, BG.

Similarly, it could be suggested that the BG of $o_{67}^c$, in which the comparison of $K_m$ values was made, might differ from normal. This issue has been fairly thoroughly settled by the group which supplied us with $o_{67}^c$. They showed that the differences, if any, are at most very minor.

A more serious difficulty arises from the fact that BG from induced E. coli has been reported to travel in at least seven discrete bands on polyacrylamide-gel electrophoresis. Formation of dimers (MW 1 million) and trimers has been tentatively suggested as a partial explanation of these isozymes. I did find that separations of all-H proteins, which gave a sharp band of ONPG-hydrolyzing activity, also showed two minor bands of activity, of about $1/3$ and $2/3$ the electrophoretic mobility of the main band; and of course by cutting out only the main band these were excluded from the turnover number determinations. But I estimate that at least $90\%$ of the BG activity was in the main band; and on gels of all-D proteins, no minor bands appeared. It therefore seems unlikely that neglecting isozymes led to significant error in the comparison of turnover numbers.
E. Synthesis of β-Galactosidase by Lac Mutants

300U: Like all other strains, this one reached a MGT of 5-6 hr after two subcultures (about 20 generations) in all-D, and even 100 generations later did not grow significantly faster in all-D. Such conclusions must be based on many experiments, since we have always found in this laboratory that, whether in H₂O or D₂O, MGT values in apparently identical experiments typically differ by 15-20%. The uncertainty expressed in the figure 5-6 hr is due to variations between experiments, the uncertainty being much smaller within a given experiment. After 10 subcultures (about 100 generations) in all-D the fully-induced P value (Figure 8) was within the range normally found in all-H, namely 3 - 6 x 10³ units. These results indicated that synthesis of BG was inhibited by D₂O to the extent typical for proteins in general and reflected in the MGT of 6 hr (cf. 2 hr in all-H). For reasons discussed elsewhere, transcription seemed the most likely site for this inhibition. The first indication that in fact transcription is not inhibited significantly came from careful measurement of the induction lag (Figure 9—same experiment as Figure 8), which turned out to be the same in all-D as in all-H, namely the well-known ⁵ 3 min at 37°. If transcription of the new mRNA for BG were inhibited as much as the growth is, it would be hard to explain the achievement of the maximal P as soon in all-D as in all-H.

To examine BG synthesis near shifts between all-H and all-D, an all-H culture was prepared by growing a tiny inoculum of all-D
Figure 8. Induction of BG by 0.5 mM IPTG in strain 300U after about 100 generations in all-D.
Figure 9. Same experiment as Figure 8, showing points early in induction.
culture through at least 20 generations in all-H. This culture was fully induced for about half a generation and then suddenly shifted into all-D + IPTG (Figure 10—the OD values after the shifts have been corrected for loss in filtering). In several attempts at this experiment, no growth resulted for hours after the all-H→all-D shift, and even in this instance growth was slow—but definitely not zero. During this slow growth in all-D, induction of BG appeared to be even more inhibited than growth (Figure 11). After 2.5 hr the cells were shifted back into the previous all-H medium. They grew with a MGT not significantly different from the previous value in all-H, and induction of BG likewise continued as though the cells had simply grown in all-H continuously.

Growth and induction of BG were followed in a shift to all-H of a culture which had grown a few dozens of generations in all-D. Figure 12 shows the growth. IPTG was added at the first arrow and P established at 2.6 x 10⁴. The cells were filtered and some suspended in inducer-free all-H, while as a control on possible harm to growth most of the cells were re-suspended in the filtrate. Figure 12 shows that the filtering and re-suspension in all-D did not slow the growth. The portion suspended in all-H grew less well than most such cultures which have just returned to all-H from all-D. There was a drop in BG activity soon after re-suspension in the inducer-free all-H. IPTG was added after 1.3 hr in all-H, and induction proceeded normally (on the high background from the previously-induced BG).
Figure 10. Growth of 300U in a double shift, all-H+all-D+all-H.
Figure 11. Induction of BG during the experiment of Figure 10.

●, first all-H; □, all-D; and ○, second all-H.
Figure 12. Growth of 300U in an all-D+all-H shift (O→o). As a control on the effect of filtering, some of the culture was re-suspended in all-D (□). IPTG was added at the arrows (†). OD₆₅₀ values have been corrected for loss in filtering.
In a shift from all-D to all-H with IPTG present throughout, there was an apparent repression by H$_2$O (Figure 14). These experiments showed that, whereas induction of BG proceeds very similarly when the steady states in all-D and all-H are compared, examination nearer to isotopic shifts reveals anomalies. Appendix II describes another which could not be repeated and is therefore not presented here as a firm result.

Although the P value many generations from a shift induced by 0.5 mM or 1 mM IPTG was the same, within experimental uncertainty, in all-D as in all-H, and the basal level likewise was the same (2 ± 1) in each medium, it was of interest to examine the efficiency of induction at intermediate concentrations. In order to study the induction uncomplicated by the effects of the galactoside pump, which can concentrate inducer into the cells by about two orders of magnitude, is itself inducible, and therefore gives autocatalytic BG induction graphs of no simple connection with the external concentration of inducer, it is necessary to use a y$^-$ strain. This was first done by Herzenberg, who found that in the y$^-$ strain which he studied P was maximal at 0.4 mM IPTG and near minimal at .04 mM. Preliminary examination of 300U after re-adaptation from all-D to all-H showed that, by contrast with Herzenberg's results, P was still nearly maximal even at .01 mM IPTG. This immediately excited suspicion that the culture was now y$^+$, which would be readily understandable in view of its having grown several generations on lactose during the adaptation to D$_2$O. However, three independent tests showed that this was not the case. (1) In the standard BG assay, if toluene
Figure 13. Induction of BG at the second arrow of Figure 12.
Figure 14. Growth (upper) and BG induction in an all-D→all-H shift, with IPTG present before and after the shift.
was omitted, the hydrolysis of BG went at 0.2 the rate of a tolue-
nized sample. This is not within the range of values normally found
for a culture with a functional y gene (Mary Brewer, personal
communication). (2) An induction at a very low concentration of
IPTG (Figure 15) did not give the autocatalytic curve which would
result\(^\text{25}\) if the galactoside pump were working. (3) On a batch of
EMB-lac plates which gave all dark colonies with an authentic y
strain, \(10^4\) colonies of 300U were grown, and every one was light pink.
It was concluded that growth in D\(_2\)O-lactose, followed by many genera-
tions in inducer-free all-D, had increased the sensitivity of in-
duction but not by activation of the galactoside pump. Workers
whom I supplied with an inoculum of my re-isolated 300U before it
had been in D\(_2\)O found in a very careful study\(^5\) that P was only about
double the basal level at .01 mM IPTG, in agreement with Herzenberg's
results and in contrast with mine on the same culture after the
growth in D\(_2\)O.

To measure the variation of P with [IPTG] in a given medium, a
culture which had been exponential for two generations was split
into five pre-warmed flasks and to each was added the appropriate
volume of 50 mM or 0.5 mM IPTG. Growth and BG production were fol-
lowed in each for at least one generation, to give the P value. The
error bars shown on the results (Figure 16) represent the limits of
the experimental scatter of each P value, and indicate that there
is some uncertainty in the factor of difference between the all-D
and all-H P values; but there is no doubt that at submaximally-inducing
concentrations of IPTG the all-D cultures were more efficiently induced.
Figure 15. Induction of BG in 300U by a sub-maximally inducing concentration of IPTG.
Figure 16. Dependence of $P$ on [IPTG] in all-D (☐) and all-H (○) 300U.
After about 120 generations in all-D, 300U was found to have lost almost all ability to make BG activity, even in 1 mM IPTG. The defect persisted after two subcultures of growth back in all-H: Figure 17 shows a P value typical for uninduced wild types, and little if at all above the culture's basal level. (In such extremely low-level BG assays, a systematic error could easily shift the whole curve so that, whereas the true graph went through the origin, the measured graph went through some other value on the OD$_{650}$ axis.)

Re-adaptation to H$_2$O on glycerol instead of acetate gave a very different result. Induced after 10 and 20 generations in H$_2$O-glycerol, the P values were respectively $2.5 \times 10^3$ and $3 \times 10^3$, uninduced P values being about 1.

The pronounced differences between acetate and glycerol were reversible, as summarized by the accompanying diagram. An arrow symbolizes one subculture in the medium in which the induced P at the head of the arrow was then measured.

The first question to settle was whether the lack of BG activity was due to inactivation or repression; that is, whether the low P
Figure 17. Failure of 300U to produce BG activity above basal after about 120 generations in all-D followed by 20 generations in all-H.
values in acetate cultures resulted from synthesis of a changed BG which had very little activity, or from a greatly decreased rate of synthesis of BG. This was answered by gel electrophoresis of soluble proteins from sonicated cells. In Figure 18B we see the induction of active BG in H₂O-glycerol. The prominent protein which appears on induction has a similar mobility to that reported in similar gel electrophoresis experiments with pure BG:¹⁰ travels in the same region as BG activity detected directly on my gels by ONPG hydrolysis; and is a considerable fraction of the soluble protein. We may therefore be confident that it is BG. In Figure 18D and 18E a similar protein is heavily induced by IPTG in those cultures (in both D₂O and H₂O) which do not produce BG activity. The loss of inducibility for BG activity is therefore due not to a failure to make BG protein, but to inactivity of the protein which is made.

Examination of Figure 18 also suggests that all-H and all-D cells differ somewhat in the quantitative distribution of soluble proteins.

Cavalli: Adapted to D₂O on acetate throughout, and then grown about 30 generations in all-D, this inducible strain showed a normal basal P, induced to 3 ± 1 x 10³. Similar values were obtained after re-adaptation to all-H through two subcultures. These findings agree with those on the other inducible strain, 300U, in which loss of inducibility came only after 120 generations in all-D.

It was suggested above, on the basis of induction lags, that transcription may not be significantly inhibited by D₂O. To examine
Figure 18. Densitometer scans of stained proteins on polyacrylamide-gel electrophoregrams of sonicated 300U. In each pair of scans the upper is from a culture to which no inducer had been added; the lower is from the same culture about one generation after adding IPTG (1 mM). A and B, H₂O-glycerol (induced P = 3x10³); C and D, D₂O-acetate(d₃) (no induction above basal); E and F, H₂O-acetate(H₃) (no induction above basal).
this issue more conclusively, Cavalli was adapted afresh to all-D (via the series of D$_2$O-lactose media). After about 40 generations it was found to be highly inducible. An inoculum of this all-D Cavalli was then grown through several subcultures in all-H. In attempt to distinguish between transcription and translation as sites of inhibition of protein synthesis, transcription of BG mRNA was performed in one medium (either all-D or all-H), the inducer was removed, and synthesis of BG was followed in the other medium. Everything was done in a 37° room. Cells were grown in a steady state, with stirring, to OD$_{650}$ = 0.2, and then induced with 0.5 mM IPTG. Seventy five seconds later, 20 ml of the culture was filtered on a Millipore membrane (type RAWP, 1.2 μ pores) and freed of IPTG with four 5-ml washes of fresh medium of the same isotopic content as the cells had been filtered from, but lacking IPTG. The filter was cut approximately in half and the cells were re-suspended by vigorous shaking for 20 sec, one portion in 10 ml all-H and the other in 10 ml all-D, both free from IPTG. (This method has since been published by Kaempfer and Magasanik.32) The filters were then removed from the cultures and samples taken at intervals into chloramphenicol solution for later assay of BG activity. The OD$_{650}$ of the re-suspended cultures, about 5 min after re-suspension, gave a correction factor for inaccurate division of the filter: all enzyme activities were normalized to constant density of culture by dividing by the OD$_{650}$ of the culture from which they were sampled. There is some uncertainty resulting from erratic changes (usually about a 10% drop) in the OD$_{650}$ immediately after re-suspension, but
these had died down by the time the OD measurement for normalization was taken; it is estimated that the uncertainty of culture density did not exceed 10%. It was hard to reproduce the height of the plateau of enzyme activity between different experiments. This is probably due to the total exposure time of the cells in IPTG. Incubation in the presence of IPTG was for 75 sec, but the time required for filtering and washing was impossible to standardize. Relatively small differences in this time represent significant variations in the total exposure time to IPTG, and therefore in the total yield of enzyme-forming potential. However, the comparison between translation in all-H and in all-D was consistent: mRNA made in all-D was translated with about the same efficiency in either medium (Figure 19), whereas mRNA made in all-H cannot be translated as well in D_2O as in H_2O (Figure 20). These results are qualitatively in accord with those on growth of the cells; all-D cells can grow very well immediately after a sudden shift to all-H, but the reverse shift inhibits growth severely. The main conclusion from these experiments is that all-D medium exerts an inhibition on BG synthesis independently of any effects on transcription, when all-H cells are shifted into all-D medium.

Comparison between Figures 19 and 20 recalls the important paper of Rittenberg and Borek which was discussed in the introduction. It appears that the fully-deuterated system is especially well adapted to catalyzing reactions of deuterated substrates.

The differences of Figure 20 might be explained by different rates of breakdown of mRNA, as an alternative to different rates
Figure 19. Translation in inducer-free media, all-D (□) and all-H (○), of BG mRNA transcribed during steady-state growth in all-D. IPTG was added at time zero and washed out at 1.25 min.
Figure 20. Experiment identical to that of Figure 19 except that transcription was in the all-H steady state.
of translation. The data of Figure 20 are re-plotted in Figure 21 in terms of the fraction of enzyme which has yet to appear. Also in Figure 21 are data from a repeat of the experiment of Figure 20. Assuming that mRNA breakdown is, at least roughly, first-order, one plots the logarithm of the fraction remaining against time. While the plots show some scatter, it is still possible to conclude that the inactivation of mRNA proceeds with about the same rate constant in the two media. It therefore seems that the lower amount of enzyme translated in all-D is due to a lower rate of translation rather than to a higher rate of breakdown of mRNA.

After about 90 generations in all-D, basal \( P \) was 1 ± 0.5, but the induced \( P \) was only 5 ± 2. Further subculturing in all-D finally gave, after a total of about 200 generations, complete lack of BG activity, with or without IPTG. This lack persisted after re-adaptation to all-H. The "induced" \( P \) was below the detection limit of 0.02 units. To test whether acetate alone could bring about such a change, 300U and Cavalli were grown through many subcultures in all-H, which contains acetate as sole carbon source. Checked after 140 and 320 generations, these cultures which had never been in \( D_2O \) remained fully inducible.

Part way through the loss of inducibility in all-D, namely after about 90 generations in all-D, an experiment was done in which addition of \( H_2O \) to make the medium about 1.5% \( H_2O \) caused a transient repression of BG, from which there was a spontaneous recovery 0.7 generation later. This experiment could not be repeated, owing to further loss of inducibility with subculturing in \( D_2O \), and is therefore not presented as a firm result, but is described in Appendix II.
Figure 21. mRNA decay plots of the data of Figure 20 and a duplicate experiment. Key: □, and ●, as in Figure 20; duplicated respectively by △ and ▲.
230U: This i⁻ constitutive strain, adapted to all-D directly and again by the method of training stepwise on lactose, often grew only slowly in all-D (MGT = 10-11 hr). P was $3 \times 10^3$, and even after about 200 generations in all-D, was still $2 \times 10^3$. The values after re-adaptation to all-H did not differ significantly.

$0^c_{67}^c$: This fully constitutive strain was trained up the most gradual series of D₂O media, on lactose. After about 8 generations in all-D, P (uninduced) was $40 \pm 20$. This is a very low figure for a constitutive of the i⁻ type, but is within the range found in the absence of inducer for $0^c$ constitutives. The D₂O-lactose culture was also continued through several subcultures, after which two spot checks gave P values of 655 and 659. After a total of only three subcultures in all-D, P was only 0.1 units, but was raised to 0.6 by IPTG. Transferring the cells by filtering into all-H and following the BG activity for 5 hr (2.5 generations) showed no rise in P; neither did three subcultures back in all-H. A few more subcultures in all-D resulted in an immeasurably low P. Growth was then followed in a double shift experiment in which the cells were shifted out of and then back into all-D (Figure 22). Although the extreme loss of BG activity (which had also been, relative to other strains, very fast) might be taken to indicate some kind of extreme adaptation to D₂O, the growth on re-suspension in all-H was immediate and fast. (Again we see the contrast with previous results.) Furthermore, half a generation of growth
Figure 22. Growth of $o_{67}^c$ shifted from all-D (□) into all-H (○), with a control re-suspension of some of the cells in all-D (▲); and then back into all-D (△), with a control re-suspension in all-H (●).
(1.3 hr) back in all-H did not destroy the ability to grow relatively well on re-inoculation into all-D.

This strain was originally believed to have no genetic potential for regulating BG synthesis, since its i and o genes were said to be at least largely deleted. This statement has now been corrected (ref. 12, and personal communication from J. Davies) so that $o^{c}_{67}$ is now said to be a double point mutant, i $^-$ and o $^c$. Point mutants can revert, whereas deletions cannot; but the probability of reversions by both point mutations, being the product of the independent probabilities, is a very small number, perhaps of order $10^{-12}$ per generation. It is therefore highly unlikely that in only a few dozen generations such a double mutant would regain control of BG synthesis. We may confidently expect that in this case, as in the inducible strain 300U, the lack of BG activity will prove to be due to inactive enzyme and not to lack of synthesis of the BG protein itself.

Unpublished work in this laboratory by Dr. V. Moses has shown that $o^{c}_{67}$ can lose ability to make BG activity under some circumstances even in H$_2$O. Since the correction of the genetic analysis, like the original wrong report, was unaccompanied by any experimental data, we should be cautious about interpretations of such changes as we have measured in $o^{c}_{67}$, pending the availability of reliable genetics.

2000 $o^{c}$: As expected from the ample constitutive BG content and the functional galactosidase pump, this strain grew well on
lactose. It was unusual in adapting very readily from D₂O-lactose to D₂O-acetate(d₃), the lag in growth being only about one hour, which is no more than is normally expected on shifting down from a sugar to acetate.

Before adaptation to D₂O, 2000 o°C had been shown by other work in this laboratory to be 9% constitutive, superinduced by IPTG to the maximum P typical of inducible strains. Values within a factor of two of these were found in preliminary checks on 2000 o°C after several generations in all-D and after re-adaptation to all-H. No thorough study was made, however, before 2000 o°C lost its ability to make any more than a very small amount of BG activity. After about 100 generations in all-D the uninduced P was 0.05 and addition of IPTG raised P to only about 5. Ten generations after re-inoculation into all-H similar values were obtained.

F. Synthesis of DNA, RNA and Protein after Shifts between D₂O and H₂O

Many of the results above have been on steady-state cultures, tens of generations away from an isotopic shift. However, the perturbations soon after shifts are also interesting, and some evidence has suggested (e.g. Figure 14) that control of enzyme synthesis is out of kilter soon after such shifts, whereas if one looks at enzyme synthesis far from a shift it has settled down to normal P values. Since it is in a sudden change from H₂O to 99.5% D₂O that the cells suffer the greatest disturbance, one might expect that
syntheses of macromolecules would be maximally disturbed in such conditions. However, it was decided to look not at such extreme results (some cultures surviving, as in Figure 10, while most do not), but rather at cultures which are experiencing considerable inhibition by deuterium, though not in the highest degree. Since it was already known (e.g. Figure 22) that about one generation of re-adaptation to all-H does not seriously impair potential to grow in all-D, a series of experiments examined how much growth in all-H, after a shift from the all-D steady state, was necessary to cause loss of ability to resume growth (if only slowly, but without a lag of hours) on re-introduction to D₂O. The result (Table IV) was that if the cells were allowed to grow two generations in all-H they suffered a lag of many hours on re-suspension in all-D, whereas 1.5 generations in all-H still allowed slow growth in all-D.

Table IV. Loss of ability to grow in all-D when D-adapted 300U grows in all-H for various periods

<table>
<thead>
<tr>
<th>No. of generations grown in all-H after inoculation from all-D</th>
<th>MGT immediately after returning to all-D</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>5 hr</td>
</tr>
<tr>
<td>1.5</td>
<td>ca. 20 hr</td>
</tr>
<tr>
<td>2.0</td>
<td>∞ (lag of many hours)</td>
</tr>
</tbody>
</table>
To measure the rate of synthesis of DNA by pulse incorporation of radioactive thymine into acid-insoluble material, the thymine-requiring strain Cavalli was used. Therefore the medium in which the non-radioactive culture was grown had to contain thymine. This made it hard to provide a high enough specific activity of thymine in the pulses, because although there is commercial tritiated thymine of very high specific activity, the non-radioactive thymine diluted the specific activity greatly. Furthermore, this dilution had to be made essentially constant by an excess of thymine in the growing culture from which the samples for pulses were taken, so that as the culture grew it would not significantly deplete the non-radioactive thymine. Five times the normal concentration of thymine was therefore provided in the culture. The final specific activity of thymine was thus forced to an inconveniently low value, giving total activities of thymine incorporated which were rather too low for good counting statistics. Also, uncertainties in the computation of spillover of counts between the two channels of the counter became magnified under these unfavorable circumstances, leading in particular to uncertainties in the differential rate $\Delta [\text{DNA}]/\Delta [\text{protein}]$. In an attempt to minimize these difficulties, the specific activity of phenylalanine-$^{14}\text{C}$ in the pulses containing thymine-$^{3}\text{H}$ was lowered tenfold compared with the value in the other series of pulses with uracil and phenylalanine. Since Cavalli does not require phenylalanine, it was necessary to check whether supplying external phenylalanine shuts off internal synthesis quickly enough compared with the times of the pulses. It was confirmed that pulses of 5.0,
10.0, and 15.0 min gave linear incorporation extrapolating to zero
time (Table V). (In this test far less radioactivity was used than
in the experiments themselves—only enough to give reasonable
counting statistics.)

<table>
<thead>
<tr>
<th>Pulse length</th>
<th>dpm incorporated pulse length</th>
<th>std. deviation of counting</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0 min</td>
<td>1873 dpm/min</td>
<td>1%</td>
</tr>
<tr>
<td>10.0 min</td>
<td>1947 dpm/min</td>
<td>1%</td>
</tr>
<tr>
<td>15.0 min</td>
<td>1914 dpm/min</td>
<td>1%</td>
</tr>
</tbody>
</table>

The results (Figures 23, 24) show the familiar rapid rise of
turbidity immediately after an all-D→all-H shift. RNA synthesis
appears to speed ahead of turbidity, rising about fivefold in one
hr. This effect is well known in other shifts-up, e.g. by changing
of carbon source from one allowing only slow growth to, for example,
glucose. A plausible explanation points out that in order to
sustain the new higher growth rate the cells will presumably need
to make many new ribosomes, and since 80% of E. coli RNA is ribosomal
RNA, it would not be surprising that RNA synthesis should rise
faster than cell wet weight. (How such shifts in the rate of making
Figure 23. Synthesis of RNA and protein in a double shift of Cavalli, all-D→all-H→all-D, as measured by incorporation of uracil-5-³H and L-phenylalanine-U-¹⁴C in 16 min pulses.
Figure 24. Incorporation of thymine-$^3$H and L-phenylalanine-$^{14}$C in 16 min pulses sampled from the same culture as that of Figure 23.
ribosomes are controlled is an unsolved problem, but meanwhile we can see that they are to the cell's advantage.) Rapid though the rise of RNA synthesis is, protein synthesis rises even faster (about tenfold in one hr), so that the differential rate of RNA synthesis, \( \Delta [\text{RNA}] / \Delta [\text{protein}] \), actually drops. After about one generation of growth as measured by turbidity, there is a drop in RNA synthesis, and perhaps in protein synthesis too. After the shift back into all-D, growth is very slow (MGT \( \approx 24 \) hr) and both RNA and protein are made only very slowly. The differential rate of RNA synthesis, however, appears to continue at about the same value to which it had settled down in all-H.

In the same experiment DNA synthesis (Figure 24), in sharp contrast to RNA synthesis, does not speed up until about one generation after the all-D\( \rightarrow \)all-H shift. This is in accord with the genetic work of Yoshikawa,\(^69\) who used quantitative transformation to measure rates of gene duplication when \textit{Bacillus subtilis} was shifted from D\(_2\)O to H\(_2\)O: a partial synchrony occurred after such a shift.

After the return to all-D, DNA synthesis drops to a very low rate. For reasons discussed above, uncertainties in measuring such low rates of incorporation are severe, and particularly the differential rate, which is the quotient of two small numbers, is subject to considerable errors. The apparent fluctuations in the differential rate may therefore not be real.
IV. DISCUSSION

A sudden all-H—all-D shift always inhibits growth severely. The fact that a given strain will sometimes grow slowly after some all-H—all-D shifts but will usually not grow after apparently identical repeat experiments contrasts sharply with the general trend in bacteriology: in experiments with $10^8$ or more cells, one does not expect statistical variability! The following hypotheses could be investigated.

(1) Perhaps this finding of variability is due to inhibition of some enzyme of which each cell has so very few molecules that stochastic variations are possible.

(2) It is conceivable that the occasional ability to grow without training in all-D reflected a subtle difference between batches of medium. However, it is hard to see how this could have come about, because the media are so simple. The most likely variation between batches of media was in the protium content, but this at most was a fraction of one per cent.

(3) Perhaps ability to grow in all-D after the extreme isotopic shift is dependent on the size of the inoculum—the cells might "communicate" chemically in some way.

(4) The previous history of the inoculum might also matter.
We are confident that training to all-D cannot be by selection of D-resistant mutants—it is much too fast (a few generations). But we must consider the possibility that prolonged culturing in D₂O leads to the selection of mutants. It is hard to imagine the nature of such a mutant. Cells undoubtedly carry unexpressed genes which they can call into play to meet some new situations; but there is no reason to believe that any ancestor of E. coli ever lived in >99% D₂O, and we are therefore confident that "dormant" genes for living in D₂O do not exist in E. coli before we put the cells into D₂O. After many generations in D₂O, however, some mutants might randomly arise and be selected by virtue of their new-found superior growth rate in D₂O. But since we know that D₂O inhibits many enzymes to a comparable extent, a mutant to grow faster in D₂O would presumably have to be a multiple mutant. As was reported in the introduction, there is no evidence that mutation rates are in general significantly higher in D₂O. Therefore we may take the usual 10⁻⁵ - 10⁻⁸ mutations per gene per generation as the order of magnitude of the frequency of a particular mutation in D₂O. Then, assuming that a mutant to grow faster would need modifications of, say, only 6 genes, the frequency of that multiple mutant would be about 10⁻³⁶. Perhaps, however, only one or two "master regulatory" enzymes are rate-limiting for growth in D₂O; then 100 generations might be long enough for formation and selection of a D-resistant mutant. If this has happened in my experiments, the mutants have not been such that increase of ability to grow in D₂O causes a
concomitant decrease of ability to grow in H₂O. Also, the superiority of the mutants is at most slight—MGT values after even 200 generations in D₂O have not been significantly less than those measured after only a dozen or so generations. But the crucial data which rule out the hypothesis of D-resistant mutants are typified by Table IV. After only two generations in H₂O D-adapted cells have lost all their acquired ability to grow in D₂O. The culture therefore cannot contain an appreciable proportion of D-resistant mutants. We conclude that, in my work at least, adaptation to D₂O is by physiological rather than genetic means. We are studying, then, the control of gene expression.

Orgel's theory has been a most satisfactory working model and has received considerable support from experiments which could have falsified it. The protein/DNA ratio is at least doubled after adaptation to all-D. Since in the other test of Orgel's model the protein/DNA ratio tripled after physiological adaptation to using trifluoroleucine instead of leucine, the value of 2 in my experiment seems credible. The extra-fast growth usually seen for a generation or two after the return of D-adapted cells to H₂O would be a corollary of Orgel's theory. The ability after that to return to all-D and grow, if only slowly (Table IV), is also a prediction which can be made from the theory, and is an experimental fact which is hard to explain otherwise. If one thinks only of isotopic composition of the cells, 1.5 generations of growth in all-H after the all-D→all-H shift would be expected to produce largely protiated cells which should then show the usual severe inhibition of growth when
shifted back into all-D. But if one considers instead the greater enzyme complements of the D-adapted cells in the Orgel model, it becomes reasonable to suppose that these extra-large numbers of enzyme molecules, which constitute the essence of the ability to grow in D_2O, might well persist to a significant extent after 1.5 generations in all-H.

The Orgel theory would not predict equal-de-repression of all enzymes. Some may not be significantly inhibited by D_2O, and there would therefore be little metabolic need to de-repress them. However, some such enzymes might be de-repressed willy-nilly by virtue of being in an operon which is de-repressed to provide necessary large amounts of one of its other enzymes. This might be the case if one enzyme of an operon were considerably more inhibited by D_2O than the others. Such effects could further complicate protein profiles. Furthermore, the potential for de-repression is not typified by the enormous range of BG P values, but often is over a much more modest range such as 5:1. The theory therefore predicts that the protein profile, i.e. the quantitative distribution of amounts of different proteins, will be different after adaptation to all-D. This does indeed seem to be so (Figure 18), and much future work could follow up this index of adaptation. For example, a series of protein profiles during D_2O→H_2O→D_2O shifts, and H_2O→D_2O→H_2O shifts, would perhaps tell us some details of the Orgel mechanism---how many proteins are de-repressed, and to what extent?
Synthesis of BG has been my main subject. It was studied rather thoroughly because it might (insofar as study of any one protein can) give some understanding of how D₂O inhibits growth. While BG is of course only one of the hundreds of proteins made by E. coli, its synthesis does include many of the important reactions at which D might inhibit. To make a BG molecule entails making a new mRNA, binding ribosomes to it, translating the codons through the interpreter tRNA, joining the peptide bonds, folding the polypeptides and finally forming the quaternary structure to give the tetramer of molecular weight 540,000 (ref. 10). BG can be a particularly revealing enzyme to study, for several reasons.

1. The assay is very sensitive, so that only small samples need be taken.

2. The assay is simple and precise, which permits the taking of many samples to study rather subtle and transient changes.

3. Induction and de-induction of BG can be performed at will using the gratuitous inducer IPTG, without seriously affecting growth.

4. Very large changes (a range of 10³) can occur in the differential rate of synthesis of BG, whereas if the induced P were only a few times the basal value it would be much harder to follow the responses of BG synthesis to stimuli.

One aspect of control of expression of the z gene which is less well understood than induction by IPTG but which may play a part in some of my results is catabolite repression. This is a form of control which can change P over a range of about 10³ even when IPTG
is present throughout. It is generally brought on by a shift into a medium which allows a faster growth rate. Believed to operate at both transcription and translation, catabolite repression serves to regulate P according to physiological conditions other than the presence of inducer. For example, addition of glucose to a culture growing on glycerol provides more energy (the growth rate increases) and the cell no longer needs BG, or at least needs it less, since the function of BG is essentially equivalent to providing glucose. (Of course the cell never really needs BG when growing on glycerol and induced by a gratuitous inducer, but I am here supposing that its physiological responses during gratuitous induction are at least qualitatively the same as those with lactose.) Because catabolite repression often is caused by shifts to higher growth rates, it may well be found in future that results such as those of Figures 13 and 14 are largely due to this mechanism of regulation.

It is convenient to summarize here those properties which differ, as well as those which do not, between cells adapted to all-D and cells re-adapted to all-H. Adaptation we take to mean growth through a few subcultures, achieving the definitive growth rate in that medium. Table VI makes this comparison.

To discuss first the results on BG, the comparison shows that BG synthesis is inhibited to the typical extent so that it stays approximately in step with other protein synthesis. There is no need to invoke any special regulation in discussing this. We would like to know whether BG can be induced to the same extent with respect to proteins in general in the two media, but there are serious
Table VI. Comparison between three steady states of growth.

<table>
<thead>
<tr>
<th>Property</th>
<th>(1) Value soon after adaptation to all-D</th>
<th>(2) Value after re-adaptation to all-H</th>
<th>(3) Value after &gt;100 generations in all-D</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGT</td>
<td>5-10 hr (in most cases 5-6 hr)</td>
<td>2-2.5 hr</td>
<td>5-10 hr (in most cases 5-6 hr)</td>
</tr>
<tr>
<td>Differential rate P of BG synthesis induced or constitutive (oC)</td>
<td>2-4 x 10^3</td>
<td>2-4 x 10^3</td>
<td>0-2 (no induction)</td>
</tr>
<tr>
<td>P of an i^-mutant (defective repressor)</td>
<td>2-4 x 10^3</td>
<td>2-4 x 10^3</td>
<td>2-4 x 10^3</td>
</tr>
<tr>
<td>BG induction lag</td>
<td>3 min</td>
<td>3 min</td>
<td>(no induction)</td>
</tr>
<tr>
<td>Protein/DNA ratio</td>
<td>1 unit</td>
<td>&gt;2.1 unit</td>
<td>(not measured)</td>
</tr>
<tr>
<td>Maximum OD_{650}</td>
<td>0.8</td>
<td>1.2</td>
<td>0.8</td>
</tr>
</tbody>
</table>

difficulties in trying to answer this question. Whereas the scatter in a given measurement of P is less than 20%, so that one might expect to be able to detect a difference of 50% in P between all-H and all-D, one finds in practice that replicate experiments to measure P in given conditions give values over a range such as 2-4 x 10^3 in all-D and the same range in all-H. Therefore we cannot tell whether BG synthesis stays in step with cell weight or with total protein, since these differ
by a factor of only two between the two cultures. Despite this uncertainty we can say that the synthesis of this inducible protein stays in step with general protein synthesis, within a factor of two.

Although BG synthesis, like protein synthesis in general, is slower in all-D than in all-H, the inhibition does not appear to lie in transcription. This finding is important because transcription could be viewed as a very likely site of inhibition, reasoning as follows. Even if the kinetic isotope effect per base pair in unwinding and transcribing DNA were small ($k_H/k_D$ close to unity), the total kinetic isotope effect on the turnover number of the DNA-directed RNA polymerase might be large, through compounding of the effect over many hundreds of base pairs. For example, $(1.02)^{200} = 51!$ Furthermore, in this peculiar case it may be that no compensation for a low turnover number can be achieved by increased synthesis of the enzyme, because there are only a few lac operators per cell. Only a very small number of RNA polymerase molecules may suffice to saturate these sites, and if that is the case increased synthesis of RNA polymerase would not increase the total catalysis of transcription. The first indication that inhibition of transcription is not sufficient to explain the inhibition of protein synthesis in D$_2$O was the result that the time lag between the addition of inducer and the appearance of the induced BG was not longer in D$_2$O than in H$_2$O. Recent results from two different ways of measuring the average rate of chain growth of RNA in E. coli at 37° indicate that to assemble the mRNA for the BG gene ($3-4 \times 10^3$ nucleotides) takes at least one min and perhaps
three min. This estimate assumes that the average rate of chain growth, which is of course a weighted mean over all kinds of RNA, applies to this one species of mRNA in particular. This seems a reasonable assumption perhaps, since it may well be the same enzyme which polymerizes all kinds of RNA. A more direct experiment gave the transcription time of the BG gene as 2-2.5 min. Even though translation undoubtedly proceeds without waiting for transcription to finish, my result suggests that transcription is not inhibited by anything like a factor of 2-3. If it were, the onset of the maximal P value as soon in all-D as in all-H would be hard to explain.

This inference was supported by the experiments in which cells induced for a time shorter than the induction lag, in all-H or all-D, were shifted to the other medium, free of inducer, for translation of the mRNA which had been made before the shift. The results were that whereas a given dose of mRNA made in all-D by D-adapted cells was translated into BG with similar efficiency in either medium, mRNA made in all-H medium by H-adapted cells was translated in all-D only about one-half as efficiently as in all-H. More precisely, these results tend to exclude transcription rather than confirm translation as a site of inhibition of the H-adapted protein-synthesizing system in all-D, since such steps as the formation of the tertiary and quaternary structure are not strictly part of translation. Furthermore, translation is susceptible to catabolite repression, and a sudden isotopic shift might set up that condition.
The greater sensitivity of all-D cultures to induction (Figure 16) by concentrations of IPTG which induce only sub-maximally may mean either that the binding of IPTG to the lac repressor is stronger in D_2O or that the cells which have grown in D_2O make less lac repressor. The latter hypothesis is weakened by the fact that the basal P value is the same in the two media; or at least is not different by anything like the large factor found at concentrations of IPTG around 1-3 μM. To decide conclusively between the two hypotheses will probably require purification and quantitative binding studies of the repressor. In my opinion this ought to be combined with a study by nuclear magnetic resonance of the binding of the lac repressor to inducers and to the operator. In its versatility at recognizing small molecules and sequences of bases with great accuracy, the repressor is rivalled only by the amino acid activating enzymes. In addition to the molecular explanation of the difference in sensitivity to induction between all-D and all-H cultures, these further physiochemical studies may also explain why both the all-D culture and the all-H culture obtained from it by re-adaptation to all-H were more sensitive to induction than the strain had been before growth in all-D.

Before this project began, one brief paper on induction of BG in deuterated E. coli had appeared, and during my work a fuller one was published. The first paper is hard to compare with my work, for several reasons. The strain of E. coli used was different (strain 112), and its genotype not at all specified; the carbon source was usually glycerol, sometimes lactose (both unenriched in
deuterium); measurements were made soon after inoculation from a maximally-dense, non-growing culture, so that a steady state of exponential growth did not obtain; and some conclusions were drawn which do not appear to be warranted by the data. With glycerol as carbon source, non-growing cells shifted from H₂O into fresh media of various percentages of D₂O containing the inducer methylthiogalactoside immediately grew four generations in 4 hr on 0% D₂O, and two generations in 4 hr even on >99% D₂O. This is in sharp contrast to my repeated result that all-H cells shifted into >99% D₂O usually fail altogether to grow, or show a lag of many hours. During these 4 hr "the formation of enzyme appeared to be even more sensitive to environmental deuterium than was the increase in turbidity", but the data do not appear to me to show this clearly, the graphs of growth and BG activity against % D₂O being perhaps parallel.

In an experiment where cells were similarly shifted but had been induced before the shift also, the rate of BG formation per unit of cell mass was said to be essentially the same in H₂O and in D₂O for the first 3 hr of incubation. Figure 25 shows the data, redrawn on a conventional plot as used in this thesis. The experimental uncertainty in it, as well as the fact that growth was not steady-state exponential, make it very hard to compare this result with any of mine. In a rather similar experiment, an uninduced (and probably non-growing) culture was diluted from H₂O-glycerol into both H₂O-glycerol and 90% D₂O-glycerol, each containing methylthiogalactoside. Over the next 3 hr the specific BG content (BG activity/cell mass)
Figure 25. Data of Henderson\textsuperscript{23} on induction of BG in H\textsubscript{2}O (\textbullet) and D\textsubscript{2}O (\textsquare).
rose twice as much in H₂O as in D₂O. Again this is hard to assess in relation to my work because the conditions of growth are so different from any I used. In a variation on this kind of shift, cells were incubated without inducer as a very dense (probably non-growing) 90% D₂O culture for two hours and then diluted into 90% D₂O medium containing methylthiogalactoside. The lag of BG induction was 1 hr (!) but the rate of production of enzyme was then similar to that in the other induction in D₂O, just described. This was interpreted to mean that "it is the induction process which is affected by deuterium rather than the process of enzyme synthesis". I do not see how this conclusion is drawn from the data. While the very long lag of induction seems suggestive that the induction process is inhibited, that conclusion also needs data to the effect that growth in general is not similarly inhibited (which in my experience it would be, in such circumstances). Then, too, if D₂O is said to inhibit induction, reasoning from the long lag, why was there no such lag in the induction immediately after the shift into D₂O?

The other paper on induction of BG in D₂O is more comparable with my work. The strains used were B (wild type), ML 3 (i⁺ o⁺ z⁺ y⁻) and ML 308 (i⁻ o⁺ z⁺ y⁺). Unfortunately, these are all different from those I used, but the main point is that their lac operons are of specified types.

The wild type shifted from H₂O-glucose to D₂O-lactose (both carbon sources unenriched in deuterium) showed a lag of many hours in both growth and BG activity. The growth lag was interpreted to
be "related in part to an inability to form the required amounts of BG". I find this correlation interesting but by no means a proof of the causal relationship which the authors seem to imply.

ML 3 showed long growth lags in D$_2$O except on glucose. In effort to minimize catabolite repression, carbon-limited growth was therefore arranged, by feeding glucose dropwise, giving linear rather than exponential growth. At 0.8 mM and 0.4 mM IPTG, P was 50-60% higher in H$_2$O-glucose than in D$_2$O-glucose. The apparent difference between this result and mine could be due to several causes. For example, it is not clear that growth on glucose, even when restricted to linear growth, will decrease catabolite repression more than does my alternative method of using a carbon source on which growth is naturally slow, namely acetate. Steady-state linear growth in a chemostat on limiting glucose was established over two days, and 0.05 mM IPTG added. In H$_2$O-protioglucose or in D$_2$O-protioglucose the induction kinetics were the same over one generation, in apparent contradiction to the previous result just mentioned. In D$_2$O-deuterioglucose, P did not achieve a steady value until a generation after adding IPTG. There was no close study of the induction lag—enzyme samples were taken at intervals of about 1 hr. If at the same time as the addition of IPTG the carbon source was changed from deuterioglucose to deuterioacetate, the induction lag was less, with respect to growth; but there was a growth lag of about 8 hr.

ML 308 (i$^{-}$o$^{+}$z$^{+}$y$^{+}$) under glucose-limiting conditions in a chemostat showed no significant difference of P between H$_2$O-glucose and D$_2$O-deuterioglucose.
In this paper as in the previously discussed one, there are several results on disturbances near isotopic shifts, but also a few on steady states. There do not appear to be any contradictions of my results. The steady-state result on the $i^{\text{th}}$ constitutive ML 30 is closely similar to my findings with 230U.

I have studied three kinds of steady state: (1) cells adapted to all-D after a dozen or so generations; (2) cells-re-adapted to all-H from all-D; and (3) cells which have grown many generations in all-D and then show loss of ability to produce active BG. The bulk of the work has compared states 1 and 2. This comparison has been somewhat clarified in a broad sense. Yoshikawa has found that DNA synthesis in deuterated cells is apparently now growth-limiting, because it does not occupy the whole cell cycle; and I have found that protein synthesis is inhibited by all-D, possibly at the stage of translation. Although Orgel's interpretation of the reduced growth rate and of the qualitative nature of the adaptation have received considerable support, the compensation mechanism is apparently limited, as growth in D$_2$O is never as fast as it is in H$_2$O, with acetate as the carbon source. I infer that increased synthesis of enzymes where catalytic activity is impaired only partly overcomes the impairment.

Undoubtedly the most surprising the puzzling results were the losses of ability to produce BG activity which occurred in D$_2$O. State (3) is a phenomenon of considerable mystery. The following evidence indicates that the unusual properties found after prolonged culturing in D$_2$O are not artifacts of contamination.
(1) Contamination has always been remarkably low in this laboratory. A flask of all-H medium opened to the air for several seconds and then kept at 37° usually shows no growth after weeks.

(2) The minimal media used throughout this work will not support growth of those many wild microbes which require richer media than salts-acetate.

(3) One would expect from the known difficulty of adapting organisms to D₂O that a considerable fraction of any which did contaminate all-D medium would not survive. This was borne out by attempts to contaminate all-D medium: several flasks were opened to the air for five seconds and then kept at 37°. The only contaminants which grew visibly to the naked eye were moulds in one flask.

(4) Phase contrast microscopy at 1600x revealed no difference between the 300U which had become non-inducible on all-D medium and authentic 300U.

(5) The pattern of soluble proteins shown by gel electrophoresis is on the whole not different in kind from those shown by many strains of E. coli.

(6) Four different strains showed similar loss of ability to produce BG activity. The conclusion seems clear that if the four strains which have appeared to lose their ability to produce BG activity have been contaminated, it is by coliform microbes which can grow faster than my adapted strains can in all-D. Since the loss of BG activity has not been accompanied by any significant decrease in MGT values, we may neglect this possibility.
Since BG is not thought to be needed for, nor harmful to, the utilization of acetate, and especially since even longer series of subcultures on acetate in H₂O did not produce this change, D₂O and not acetate does seem to be a necessary cause of the loss of ability to produce active BG. Reports of similar phenomena are scarce, but Hinshelwood's group has worked on very slow adaptations (taking many subcultures), and interpreted them in a rather unfashionable way which shunned the concept of selecting mutants. Insofar as Hinshelwood formulated a chemical (as distinct from mathematical) theory of long-term adaptation,¹³ he tended to predict different protein profiles after adaptation. My results with gel electrophoresis of soluble proteins do indeed appear to show such changes. However, the viewpoint stressing physiological adaptation seems to be no more helpful than the mutation-selection concept in explaining why the change occurred. The explanation may require some radically new analysis. Also the continuation of BG inactivity in H₂O-acetate is odd. Other workers in this laboratory have found (V. Moses and P. B. Sharp, unpublished results) that the protein profile as measured on gels is characteristic of the medium in which the cells are growing, and there is little "memory effect"; for example, cells which had been cultured on acetate for hundreds of generations afford the profile characteristic of growth on broth, not acetate, after only one subculture on broth. My contrasting results, in which an adaptation which occurred in D₂O persists in H₂O, but is reversed by glycerol so that we know it is not a mutation of any ordinary sort, are unusual in that a condition produced by one
environment persists for 30 generations in another environment, namely H\textsubscript{2}O-acetate, which will not itself produce that condition, but the change is simply demonstrated by the re-activation with glycerol not to be the result of a mutation. Some people would argue that this is a case of Lamarckian inheritance, but I do not think that Lamarckianism properly defined is even a meaningful concept for one-cell organisms.

It may be that glycerol, or a metabolic product of it, will re-activate the faulty BG protein even in the absence of IPTG. If so, studies on the pure protein would then be interesting.

A largely different area of work has been on states which are far from steady, soon after shifts into or out of D\textsubscript{2}O. Pulse incorporation of precursors of DNA, RNA and protein showed that these three major kinds of macromolecule can suffer great changes within minutes after isotopic shifts. Perhaps the most detailed work in this area has been by Sato,\textsuperscript{28} who has found that within a few minutes D\textsubscript{2}O greatly increases the aggregation of spindle protein in echinoderm eggs. This is one of the few specific molecular studies in the literature on biological effects of D\textsubscript{2}O. The changes I found in rates of synthesis of macromolecules of the three main kinds could not in general have been predicted from the mass growth rate (turbidity of cultures). The shift of fully-adapted all-D cells into all-H produced the most spectacular changes. RNA synthesis accelerated greatly, which I predict will be found to be owing mainly to the need to make many new ribosomes. However, protein
synthesis speeds up even more, so that the differential rate of RNA synthesis slows. Perhaps the need for ribosome-making to burst ahead of growth is now over, each cell having amassed the definitive all-H ribosome complements. In a general qualitative way, Orgel's theory will probably be a useful guide to investigating these phenomena; but their quantitative explanations will surely be very complicated. During the first generation of rapid growth after the shift all-D→all-H, DNA synthesis proceeds at approximately the same rate as before the shift, in sharp contrast to the changes in DNA and protein syntheses. However, at the end of this period there is a big burst of DNA synthesis, as had previously been measured by the very different technique of gene dosage assays by transformation.69

After the shift back into all-D, growth is very slow. It may be that more sensitive methods in future will be able to detect differential effects on one kind of macromolecule more than others, but for the present all we can say is that there is, approximately, a co-ordinate shutdown of DNA, RNA and protein syntheses.

The shift of all-H adapted cells into D₂O has received little attention in this work, because of the variability of the results and because little if any growth ensues. However, we do know that mRNA made in H₂O by H-adapted cells can be translated in D₂O, though not as efficiently as in H₂O (Figure 20). Future study of this shift might further examine syntheses of macromolecules, by pulse labeling; substrate uptake and respiration, by manometry; and
perhaps protein synthesis in general (by uptake of a radioactive amino acid, for high sensitivity) compared with synthesis of a particular protein such as BG.

Our simplest model for further work is as follows. A sudden all-H to all-D shift inhibits translation, and also other unknown reactions to a critical extent. But gradual training allows buildup of the protein/DNA ratio, and the distribution of proteins, characterizing the state of D-adaption. However, enzyme de-repression cannot fully counteract enzyme inhibitions. The balance of macromolecular composition which characterizes D-adaption is readily lost by two generations back in all-H. Quantitative macromolecular composition will be the most important kind of study in future work on the poisoning of bacteria by D₂O.

When I told Professor Donald Glaser that I was working on biological effects of D₂O he cried, recoiling, "But that must be a bottomless pit!" I tend to agree with him. However, I think it does offer interesting aspects of control of metabolism even though one may not expect to "get to the bottom of it". This survey of chemical effects of D₂O on E. coli has of course not pretended to be comprehensive; neither has it succeeded in discovering "the" reason why D₂O poisons organisms. We do now have, however, a better idea of where to look next.
REFERENCES

13. A. C. R. Dean and C. Hinshelwood, "Growth and Regulation in Bacterial Cells" (Oxford Univ. Press, 1966), Chaps. 8, 9 and 10, especially p. 274.
52. Dr. S. Person (Penn. State Univ), personal communication.
APPENDIX I

Assay of $H_2O$ in $D_2O$

Since even 0.2% $H_2O$ in 99.8% $D_2O$ is 0.1 M, and since such "small" concentrations of $H_2O$ may affect the synthesis of proteins in highly-deuterated bacteria, an analysis for $H_2O$ in $D_2O$ is needed. Nuclear magnetic resonance of the protons (pmr) is a suitable method because it is specific, sensitive, non-destructive, and fast.

The integrated pmr is proportional to the concentration of $H_2O$ (actually HDO) if these precautions are taken:

1. The concentration of paramagnetic species must be constant. This means that either all $O_2$ must be removed or else, what is easier, all solutions must be in equilibrium at room temperature with air. Also, paramagnetic metal ions, present in most bacterial culture media, must be at the same concentrations in reference and unknown solutions.

2. The temperature of reference and unknown samples must be the same.

3. The internal diameter of sample tubes must be identical. This is because the instrument "sees" only a few mm length of the tube and the volume of sample in that length will be constant only if different tubes have the same internal diameter.
(4) Exposure to air (which contains $\text{H}_2\text{O}$ vapor) must be minimized. If the pmr tubes are not sealed—e.g. with grease—the $\text{H}_2\text{O}$ content of the $\text{D}_2\text{O}$ slowly increases.

The integrated pmr was calibrated by addition of known concentrations of $\text{H}_2\text{O}$ to the $\text{D}_2\text{O}$. This can be done by weighing, but an easier way is to make a 5% solution of $\text{H}_2\text{O}$ in the $\text{D}_2\text{O}$ and add that volumetrically to known volumes of the $\text{D}_2\text{O}$. Hamilton micro-syringes are very convenient for this. 0.5 ml final volume suffices.

Integration of the pmr is more precise if spinning sidebands are minimal and if the water resonance is swept through quickly so that drift contributes less than it would have at longer sweep times.

It would seem desirable to extrapolate each integrated pmr to zero RF field, but the signal:noise gets so low at RF fields below about 0.1 mG that this is useless.

The molarity of $\text{D}_2\text{O}$ is $18/20 = 10\%$ less than that of $\text{H}_2\text{O}$, but the density is 1.105 at $25^\circ\text{C}$, so that volume fraction = mole fraction in mixtures of $\text{H}_2\text{O}$ and $\text{D}_2\text{O}$.

Figure 1 shows a typical analysis.

References

1. Appendix II.
Appendix I, Figure 1. Slope = 31 units per 1% H₂O. Therefore 18 units at zero added water corresponds to 0.6% H₂O (99.4% D₂).
APPENDIX II

Repression of BG by a Small Concentration of $H_2O$

Part way through Cavalli's loss of ability to produce BG activity (after about 90 generations in all-D), an exponential all-D culture was induced 1 mM IPTG to a P value of 5. Distilled water (pure $H_2O$) was then added to 1%. This had no discernible effect on the growth, but did cause a severe repression of BG, from which a spontaneous recovery occurred after 0.7 generation. This is the kind of response, known as catabolite repression, which is well known in shift-up experiments such as addition of glucose to a culture growing on acetate. For comparison, an example of a catabolite repression is shown. Other more precise examples can be found in ref. 51 of this thesis.

One percent $H_2O$ did not repress fully-inducible all-D cultures. The experiment reported in this appendix was not repeated because soon after it was done Cavalli became completely uninducible.
Appendix II, Figure 1. Repression by H₂O, 0.20 ml added at the arrow to a 20 ml induced culture of Cavalli. MGT throughout, 5 hr.
Appendix II, Figure 2. Catabolite repression of Cavalli (all-H) growing on acetate. IPTG (1 mM) was added at the first arrow; glucose (10 mM) at the second arrow.
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