The realization that the synthesis of proteins is often under the control of repressors\(^1,^2\) has posed a central question in molecular biology: What is the nature of the controlling substances? The scheme of negative control proposed by Jacob and Monod envisages that certain genes, regulatory genes, make products that can act through the cytoplasm to prevent the functioning of other genes. These other genes are organized into operons with cis-dominant operators, such operators behaving as acceptors for the repressor. Appropriate small molecules act either as inducers, by preventing the repression, or as corepressors, leading to the presence of active repressor. The simplest explicit hypothesis for inducible systems is that the direct product of the control gene is itself the repressor and that this repressor binds to the operator site on a DNA molecule to prevent the transcription of the operon. The inducer would combine with the repressor to produce a molecule which can no longer bind to the operator, and the synthesis of the enzymes made by the operon would begin. However, other models will also fit the data. Repressors could have almost any target that would serve as a block to any of the initiation processes required to make a protein. A molecular understanding of the control process has waited on the isolation of one or more repressors.

We have developed an assay for the lactose repressor, the product of the control gene (i gene) of the lactose operon. The assay detects and quantitates this repressor by measuring its binding to an inducer, as seen in this case by equilibrium dialysis against radioactive IPTG (isopropyl-thio-galactoside).

In order to seek the lactose repressor, we desired some means that would not depend on the specific models that might be imagined for the actual mechanism of repression. The minimal assumption on which the assay is based is that there should be some interaction between the repressor and the inducer. However, inducing substances added to the cell are often modified before they can trigger induction. Such is the case with lactose which must be split by \(\beta\)-galactosidase in order to induce,\(^3\) but the thiogalactosides appear to be true gratuitous inducers; no chemical modification has yet been detected associated with their action as inducers. The ability of IPTG to stabilize certain temperature-sensitive i-gene mutants\(^4\) argues strongly that the i-gene product interacts directly with this inducer. Furthermore, the existence of a competitive inhibitor of induction, ONPF (o-nitrophenyl-fucoside), which can also stabilize certain leaky i mutants and which behaves as though it drives the repressor into the form that shuts off the operator, also supports this thesis.\(^5\)

Design of the Experiment.—In order to detect the binding of IPTG to the repressor by equilibrium dialysis, one must achieve concentrations of repressor that are comparable to the dissociation constant of the complex. What affinity does the repressor have for the inducer? Half-maximal induction occurs at \(2 \cdot 10^{-4} \text{ M}\) IPTG in a permeaseless strain. This fact alone does not lead directly to an estimate of the equilibrium constant because the enzyme level changes by a factor of 1,000 on
induction. Since the rate of enzyme synthesis varies inversely with the first power of the repressor concentration, the concentration of free repressor must have dropped by a factor of 1,000. Because the interaction between the repressor and inducer is quadratic in the inducer concentration, as is shown by the shape of the induction curve, the inducer concentration must be a factor of the square root of 1,000 above the equilibrium constant at half-maximal induction. Thus, the wild-type repressor should have an affinity of the order of $6 \cdot 10^{-6} \text{M}$ for IPTG, and if any binding is to be detectable, the repressor concentration must exceed a few times $10^{-6} \text{M}$. Since the cell pellet is about $10^{-9} \text{M}$ in cells, and since one does not expect many copies of the repressor per operator site, it seems likely that one would have to fractionate and concentrate the repressor by at least a factor of 100, working blindly, before detecting any effect. To improve the chances for success, we decided to isolate a mutant bacterium in which the $i$ gene produces a product that binds more tightly to the inducer. Fortunately, such a mutant, an $i^t$ (tight-binding) mutant, was found.

The $i^t$ Mutant.—We enriched for an $i^t$ mutant by using a technique which will select preinduced cells out of an uninduced population. A challenge with a very low level of inducer will trigger only those cells that are unusually sensitive. Such cells will be selected for along with constitutive mutants, but the constitutives can then be selected against by growth in the presence of TONPG, a compound that inhibits cells with an expressed permease (see Experimental Details). A super-inducible mutant was found. The induction curve of a permeaseless derivative of this strain is shown in Figure 1. The basal level is raised, in comparison to the wild type, but the midpoint of the induction curve is pulled lower than would be expected on the basis of the changed basal level alone. Furthermore, in contrast to the wild type, the induction kinetics are linear at the lowest levels of inducer (shown in the insert), the basal level being doubled at $7 \cdot 10^{-7} \text{M}$ IPTG, and the behavior only becoming quadratic as the level of inducer rises toward that necessary for full induction. The linear behavior suggests that this range shows single-site binding of IPTG to the repressor and that the point at which the basal level is doubled corresponds to the condition that half the repressor is bound to IPTG and half is free to repress. This yields a naïve estimate for the $K_m$ of $7 \cdot 10^{-7} \text{M}$. The rest of the curve can be interpreted as showing that there are two (or more) sites for the binding of IPTG on the molecule and that in order to scavenge the last repressor

### TABLE 1

**The Reversible Binding of C$^{14}$ IPTG**

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>Cpm/0.100 ml</th>
<th>Corrected value</th>
<th>Excess bound as % of outside concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outside concentration of C$^{14}$ IPTG</td>
<td>475</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1—Inside after 30 min dialysis</td>
<td>841</td>
<td>1025</td>
<td>116</td>
</tr>
<tr>
<td>2—Inside after 1 hr dialysis</td>
<td>950</td>
<td>1125</td>
<td>137</td>
</tr>
<tr>
<td>3—Inside a sac after 1 hr further of dialysis against buffer without IPTG</td>
<td>78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4—Inside after another 30 min dialysis against C$^{14}$ IPTG</td>
<td>795</td>
<td>1035</td>
<td>118</td>
</tr>
</tbody>
</table>

Four identical 0.1-ml samples of protein were dialyzed against C$^{14}$ IPTG in TMS buffer at 4°. At the end of 30 min, the IPTG concentration inside sac #1 was measured. At 1 hr the concentration inside sac #2 was measured, and the others were transferred to unlabeled buffer. At the end of the next hour, sac #3 was read, and sac #4 was transferred back to the original flask for another 30 min. The raw data, cpm/0.100 ml, are given in the first column. The volume of each sample was measured, to correct for the water uptake during the dialysis and that carried on the walls of the dialysis sac, and the corrected numbers, expressed as cpm/0.100 ml, are given in the second column.
off the operator, both IPTG sites must be fully loaded. (If the two-site nature of the curve is taken into account, the estimate for the binding constant would rise to about $1.2 \cdot 10^{-6} \text{M}$.)

Detection of an Effect.—Since, even for the mutant strain, the repressor must be more concentrated than it is in the cell in order to display any binding of IPTG, we made a diploid derivative of the $i^r$ mutant strain and proceeded to fractionate cell extracts with spermine and ammonium sulfate and to examine the concentrated protein fractions. The first detection of any effect was marginal; the spermine precipitate from the mutant strain yielded a 4 per cent binding ($1000 \text{cpm/0.1 ml}$ outside a dialysis sac, $1040 \text{cpm}$ within). Further purification, however, immediately produced greater effects. The procedure described in the Experimental Details yields material that will draw the inducer into a dialysis sac to a concentration 1.5–2 times that outside the sac (50–100% excess binding) at a protein concentration of 10 mg/ml.

The labeled IPTG is bound reversibly; it can be freely dialyzed into, out of, and even into the sac again. This is illustrated in Table 1. At $4^\circ\mathrm{C}$, the dialysis goes essentially to completion in about 30 minutes; assays were run for periods ranging up to eight hours. That the observed binding is not due to a contaminant in the radioactive IPTG was confirmed by using two different preparations of labeled IPTG (a C$^{14}$-labeled commercial preparation and a H$^3$-labeled Wilzbach preparation) and by examining the competition with unlabeled IPTG.

Negative Controls.—Is the material that binds IPTG really the $i$-gene product? The most critical controls are to examine a diploid amber-suppressor-sensitive $i^-$ strain, which should have only a fragment of the $i$-gene product, and to examine a diploid $i^a$ strain, which should have an $i$-gene product that is unable to recognize the inducer. The $i^a$ strain that was used, like many such strains, is slightly inducible at 0.1 M IPTG. This affinity of the $i^a$ repressor for IPTG should be completely undetectable by the assay. The necessary diploid strains, isogenic to the $i^r$ mutant strain, were constructed by F-duction. An identical parallel purification was run on 50-gm lots of control cells and $i^r$ mutant cells. At the end of the purification, samples of each protein solution were dialyzed against several different IPTG concentrations. Figure 2 shows the result for the $i^a$ control; no binding was observed. This finding implies that the substance to which the IPTG binds is either the $i$-gene product or else some other material of the lac operon whose synthesis would be blocked in this strain. This second possibility is ruled out by the control shown in Figure 3. No binding was found with the fraction isolated from the $i^-$ strain. Since this strain is wild type with respect to the lactose enzymes, no binding to any of them was being observed.

Other less specific negative controls have been done. No binding was found in an isogenic diploid deletion strain carrying the Beckwith deletion M116, which has cut out the $i$ gene as well as the beginning of the $\beta$-galactosidase gene. Mixing experiments were done with the extracts from this deletion and the sus $i^-$ strains mixed with the $i^r$ extract to show that no inhibitors were present in the negative controls. Furthermore, we examined, in haploid strains, a deletion of the entire lac region carried in the Lederberg strain W-4032 and the $i^-$ strains ML 308 and 2.340; no binding was found. The haploid amount of wild-type $i$-gene product is detectable. Our yields, however, are not sufficiently consistent to guarantee a factor of two
The induction of β-galactosidase in the $i^+$ mutant. A permease-negative derivative of the $i^-$ mutant, carrying an $F^+ gal^+$ factor, was grown at 35°C in M56-glycerol medium for ten generations in the presence of various concentrations of IPTG. The β-galactosidase activity was measured on toluenized samples. The insert shows that the induction kinetics are linear at the lowest concentrations of inducer. The open and closed circles represent independent experiments done with different permeaseless derivatives.

The binding ability of an $i^+$ strain. The repressor fraction was isolated in parallel from 50-gm lots of isogenic $F^+ i^+$/ and $F^+ i^-$/ cells. Samples of each extract were dialyzed against different concentrations of C$^{14}$ IPTG for 8 hr at 4°C in TMS buffer as described in the Experimental Details. The excess label bound inside each dialysis sac, normalized to a protein concentration of 10 mg/ml, is plotted against the IPTG concentration outside the sac. The corrections for protein concentration are of the order of 20%.

The binding ability of a suppressible $i^-$ strain. The repressor fraction was isolated in parallel from isogenic $F^+ sus^+/sus^+$ and $F^+ i^-/i^-$ cells and assayed as described for Fig. 2.

The binding constants of the wild-type and $i^+$ mutant strains. Samples isolated from isogenic wild-type and $i^+$ diploid strains were dialyzed against many different concentrations of C$^{14}$ IPTG as described under Fig. 2. The amount of IPTG bound at each external IPTG concentration, normalized to a protein concentration of 10 mg/ml, is plotted against the ratio of bound to free IPTG. Single-side binding should obey the relation: (Amount bound) = (number of sites) − $K_m$ (bound/free).
difference that might be due to gene dosage, so we cannot usefully compare haploid and diploid strains.

The amount of repressor is not increased if the cells are fully induced. Neither is the purification affected if done in the presence of a high concentration of IPTG.

**Positive Controls.**—Since the binding of IPTG to the wild-type repressor is detectable, the binding constants of the $i^+$ mutant and wild-type strains can be compared *in vitro*. By measuring the amount of IPTG bound at various IPTG concentrations and plotting the amount bound against the ratio of bound to free IPTG, one would get a straight line if there is a single type of noninteracting binding site. The intercept on the $y$ axis will be the molarity of binding sites and the slope will be the negative of the binding constant. Such plots are shown in Figure 4 for the mutant and wild strains. The binding is linear, and the measured binding constants ($K_m$) *in vitro* at $4^\circ$C are $6 \cdot 10^{-7} M$ for the $i^+$ mutant and $1.3 \cdot 10^{-6} M$ for the wild type. The *in vivo* estimates led us to expect that these binding constants would differ by a factor of 4–10. We find only a factor of two difference and can only suggest that temperature effects, buffer effects, or the indirect nature of the *in vivo* estimates might account for this discrepancy. The important finding is that the mutation in the $i$ gene that produces the superinducible phenotype, and presumably a repressor more sensitive to inducer, alters similarly the substance observed in vitro.

What affinities does the binding site have for other compounds? One can measure $K_i$'s by dialyzing the repressor against a mixture of radioactive IPTG and varying amounts of unlabeled competitors. Such estimates are compiled in Table 2. Competition with cold IPTG yields essentially the same binding constant as was obtained with the labeled IPTG. This confirms that it is truly the IPTG that is binding. TMG (thio-methyl-galactoside), which is a weaker inducer than IPTG, has a weaker binding constant. ONPF, which is a competitive inhibitor of induction (and behaves as though it drives an allosteric equilibrium toward the form that binds to the operator), shows a 40-fold weaker binding than IPTG, again about the value that would be anticipated from its behavior *in vivo*.8

The interaction with galactose is of interest because the $i^+$ repressor *in vivo* shows a sensitivity to galactose. The enhanced basal level of $\beta$-galactosidase in the $i^+$ strain is doubled if the $i^+$ gene is in a strain that lacks galactokinase. Such strains have internal galactose pools of the order of $2.5 \cdot 10^{-4} M$ produced from UDPGal.9 Thus one expects an interaction between the mutant repressor and galactose in this concentration range.

Glucose shows very little affinity for this site. There is a 100-fold discrimination between glucose and galactose. The magnitudes of all these binding constants further support the identification of this material as the repressor.

**Properties of the Repressor.**—The ability to bind IPTG is not attacked by RNase or DNase. It is destroyed by pronase. The binding site can be inactivated by temperatures above $50^\circ$C. These are properties of the part of the molecule that interacts with the inducer. As yet there is no way of seeing the rest of the molecule.

### Table 2

<table>
<thead>
<tr>
<th>Competitor</th>
<th>$K_i$ for the $i^+$ gene product</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPTG</td>
<td>$5 \cdot 10^{-7} M$</td>
</tr>
<tr>
<td>TMG</td>
<td>$2 \cdot 10^{-6} M$</td>
</tr>
<tr>
<td>ONPF</td>
<td>$2 \cdot 10^{-6} M$</td>
</tr>
<tr>
<td>Galactose</td>
<td>$5 \cdot 10^{-4} M$</td>
</tr>
<tr>
<td>Glucose</td>
<td>$\geq 3 \cdot 10^{-2} M$</td>
</tr>
</tbody>
</table>

*Binding constants inferred by competition of the unlabeled material with labeled IPTG. Protein samples were dialyzed against $1.2 \cdot 10^{-7} M$ C14-IPTG and three different concentrations of each of the competitors for 2 hr at $4^\circ$C. The amount of IPTG bound was measured, corrected for any changes in the protein concentration, and plotted to permit an estimate of the $K_i$.***
Fig. 5.—Sedimentation of the repressor. Samples of 0.2 ml of a concentrated repressor fraction were layered on 5-ml gradients, from 5 to 30% glycerol, in TMS buffer. The gradients were spun for 8.5 hr at 50,000 rpm at 4°C. In one tube, catalase and β-galactosidase were run as sedimentation markers. For the experiment shown in (a), the repressor fraction was brought to 2.5-10⁻⁷ M C¹⁴ IPTG and layered on a gradient containing 1.5-10⁻⁷ M C¹⁴ IPTG. After the centrifugation, two-drop samples were collected directly into scintillation vials and counted with Bray's solution. The background level in the gradient is about 1000 cpm; the counts are plotted as a per cent of the background level (open circles). For the experiment shown in (b), a parallel gradient was run without IPTG. The optical density was measured on each two-drop sample at 260 mµ (— — —) and 280 mµ (———). Three-tube samples were pooled and placed in dialysis sacs. Their volumes were reduced by burying the sacs under dry G 200 Sephadex for 1 hr. Then the samples were dialyzed against C¹⁴ IPTG. The excess bound, corrected to 0.100 ml final volume inside the dialysis sac, is plotted as a bar. Superimposed on both experimental curves is the position of the markers taken from a parallel gradient.

The sedimentation of the repressor was followed on glycerol gradients. By sedimenting the material through a gradient in the presence of a uniform level of labeled IPTG, one can observe a peak of label bound in equilibrium. This is shown in Figure 5a. In a parallel tube, catalase and β-galactosidase were used as markers. The repressor sediments at 7–8S; we estimate its molecular weight to be 150,000–200,000. Figure 5b shows the repressor seen by assaying after a gradient without IPTG. The tubes were pooled; the samples were concentrated and dialyzed against radioactive IPTG. The 280- and 260-mµ absorption is indicated, to underline the impurity of our preparation. The material that binds IPTG sediments again at 7–8S.

We estimate the amount of repressor in the cell by the yield that is obtained after the first steps of purification. The most recent procedure, breaking the cells with glass beads in a Waring Blender, and fractionating directly with ammonium sulfate, yields all of the binding ability in the 0–35 per cent cut. The highest recoveries are about 100 sites per cell for the binding of IPTG. These are diploid cells in a tryptone-yeast extract medium harvested late in the log phase. These cells may have four to six gene copies, so we interpret the figure as 20 sites per gene copy and
probably ten molecules per gene if there are two sites for the inducer on each molecule. With this last assumption, the repressor corresponds to about one part in $10^4$ of the cell’s proteins.

Conclusions and Outlook.—Our findings that the $i$-gene product is a protein, that it is uninducible, and that it occurs in a small number of copies serve to confirm many of the expectations that have grown up over the years. The discovery of temperature-sensitive mutants in the $i$ gene implied that the $i$ gene coded for a protein. The isolation of amber-suppressor-sensitive $i^-$ mutants further proved the point.

The estimate of a small number of copies of the repressor has been the traditional explanation of the phenomenon of escape synthesis. The positioning of the $i$ gene outside the operon and an in vivo experiment on $i$-gene induction both argue that the level of the $i$ product would not rise and fall with the state of induction of the lactose enzymes.

An explicit assay, however, unambiguously demonstrates these points and opens the way to a full physical and chemical characterization of the $i$-gene product. Furthermore, experiments designed to ask which steps are blocked by the repressor are now possible in vitro.

Summary.—The lac repressor binds radioactive IPTG strongly enough to be visible by equilibrium dialysis. This property serves as an assay to detect the repressor, to quantitate it, and to guide a purification. It is a protein molecule, about 150,000–200,000 in molecular weight, occurring in about ten copies per gene. That the assay detects the product of the regulatory $i$ gene is confirmed by the unusually high affinity shown for IPTG, by the difference in affinity of the substances isolated from the wild-type and a superinducible $i$-gene mutant, and by the absence of binding in fractions from $i^-$, $i$-deletion, and $i^+$ strains.

Experimental Details.—Buffers: Two buffers were used. TMEM buffer contained 0.01 M tris HCl, pH 7.4, 0.01 M Mg$^{++}$, 0.006 M $\beta$-mercaptoethanol, and $10^{-4}$ M EDTA; TMS buffer was 0.2 M KCl in TMEM buffer. Double-distilled water was used throughout.

The assay: A 0.1-ml protein sample was dialyzed at 4°C with shaking against 10 ml of TMS buffer containing $1.2 \times 10^{-7}$ M C$^{14}$ IPTG (about 500 cpm/0.1 ml). Number 20 visking tubing was prepared by boiling three times in $10^{-3}$ M EDTA and stored in $10^{-4}$ M EDTA. A sac about 3–5 cm long was used for the dialysis. After the dialysis had gone to completion, the contents of the sac were squeezed out into a tube. A 0.100-ml portion was put directly into Bray’s solution and counted in a scintillation counter. A standard was made by taking a 0.100-ml sample of the external fluid. An additional 0.020 ml of the dialyzed protein sample was used for Biuret determination of the protein concentration, to correct for variations in liquid uptake from sample to sample. C$^{14}$ IPTG, 25 mC/mM, was obtained from Calbiochem.

The Purification: Cells were grown in 8 gm Tryptone, 1 gm yeast extract, and 5 gm NaCl per liter of medium. They were harvested in late log phase at $1.5 \times 10^9$ cells/ml. The cultures were poured on ice; the cells were washed in TMEM and stored frozen in 50–60-gm lots. For most of the experiments described in this paper, the following purification was used. A 50-gm lot of cells was broken in the Hughes Press, and taken up in 200 ml of TMEM. After a DNase treatment (Worthington, electrophoretically pure) at 2 $\mu$g/ml and a low-speed spin, the extract was brought to 2 mg/ml with spermine. The spermine precipitate was dissolved in 50 ml of TMS buffer and the ribosomes were removed by a 90-min, 40,000-rpm spin. The high-speed supernatant was brought to 35% saturation with solid ammonium sulfate, at pH 7.0, and the precipitate collected, dissolved in, and dialyzed against TMS buffer. All these operations were carried out at 4°C.

Our latest procedure consists in breaking the cells by blending with glass beads in a Waring Blender. Cells (150 gm) are blended with 150 ml of TMS buffer and 450 gm of glass beads for 15 min. The extract is made up to 5 vol with TMS buffer. After a DNase treatment and a low-speed spin, two ammonium sulfate precipitations are done: the 0–23% cut is discarded; the
23–33% cut is saved. The repressor is then applied to a DEAE Sephadex column in 0.075 M KCl in TMEM and eluted during a gradient at 0.15 M KCl.

Selection of the $v$ mutant: A Sm$^R$ derivative of W3102 ($F^-\text{gal k}^-$), obtained from M. Meselson, was used. After mutagenesis in N-methyl-N'‑nitro-N-nitrosoguanidine (100 µg/ml in mineral medium M56 for 1 hr at 37°C) and segregation, the cells were given a maintenance challenge. They were grown in M56 with $10^{-3}$ M glycerol, $8\cdot 10^{-4}$ M leucine, and 10 µg/ml B$_2$ at 42°C for six generations, challenged with $10^{-6}$ M IPTG during the last two generations of growth, then chilled at $5\cdot 10^5$ cells/ml, washed twice with preconditioned medium (made by growing W3102 to glycerol exhaustion in M56), and inoculated at $5\cdot 10^6$ cells/ml into 100 ml M56, preconditioned, containing $10^{-3}$ M melibiose and $1.5\cdot 10^{-3}$ M ONPF. 13 After 36 hr the bacteria came up, about 50% constitutives. For the back selection, the bacteria were deadapted in M56-glycerol, then inoculated at $10^6$/ml into M56 containing $3\cdot 6\cdot 10^{-3}$ M TONPG which, as we found, inhibits the growth of bacteria whose lac permease is expressed. After three cycles of forward and backward selection, the bacterial colonies were screened with ONPG on plates containing $10^{-6}$ M IPTG.

Enzyme assays: The lactose enzymes were assayed as described previously. 8

We wish to thank Christina Weiss and Susan Michener for their technical assistance; Drs. Jonathan Beckwith, Salvador Luria, and Matthew Meselson for providing bacterial strains; and the National Institutes of Health (GM 09541-05) and the NSF (GB 4069) for their support of this work.

Abbreviations used: IPTG, isopropyl-l-thio-β-D-galactopyranoside; ONPF, o-nitrophenyl-β-D-fucopyranoside; ONPG, o-nitrophenyl-β-D-galactopyranoside; TMG, methyl-l-thio-β-D-galactopyranoside; TONPG, o-nitrophenyl-l-thio-β-D-galactopyranoside.

9 Wu, H. C. P., and H. M. Kalckar, manuscript in preparation.