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The Specific Effectors of Viral Development

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The development of wild strains of poliovirus is inhibited by a number of substances. Among them is guanidine. From an original guanidine-sensitive strain, guanidine-resistant mutants are easily obtained. And from these guanidine-resistant mutants it is possible to select guanidine-exacting variants. Thus, for the poliovirus, guanidine is an inhibitor, or is devoid of action, or is indispensable. Moreover, guanidine blocks neither cellular growth nor cellular multiplication. Guanidine acts on the virus.

Our problem is that of guanidine considered as a specific effector of the development of the poliovirus. Guanidine, however, is only one effector among others which will be discussed. This will lead us to envisage in its generality the problem posed by the specific effectors of viral development.

The guanidine-sensitive strain

The poliovirus, like any other virus, is an obligate intracellular parasite. To study its development, animal cells are infected and suspended in a nutrient medium. The cells are gently aerated at the optimum temperature (about 36°). Under standard experimental conditions the life cycle of the poliovirus lasts about 7 hr. During the first 3 hr. the viral genetic material does not multiply. This is the primary or latent phase. Then the viral nucleic acid starts multiplying and this marks the beginning of the secondary phase. The proteins of the coat are synthesized. The nucleic acid condenses and is enclosed in a proteinic shell, the capsid. The viral particle, the virion, is thus formed. The infected cell eventually bursts, liberating about 100 000 virions, of which only 50–3000 prove to be infectious under experimental conditions.

If the medium is supplemented with 1 mM-guanidine, the viral cycle is blocked. The viral nucleic acid does not multiply and, of course, virions are not produced. How does guanidine act?

In the animal cell, ribosomal RNA, messenger RNA and transfer RNA are built on structural genes acting as a template. The animal cell possesses

enzymes that synthesize RNA on a DNA template, but is devoid of enzymes capable of replicating RNA. The genetic material of the poliovirus is RNA. To replicate its genetic material the poliovirus has therefore to produce a new enzyme, an RNA replicase.

An RNA replicase is in fact present in the infected cell (Baltimore & Franklin, 1963; Baltimore, Eggers, Franklin & Tamm, 1963). This enzyme can synthesize RNA on an RNA template *in vitro* as well as *in vivo* (Baltimore, 1964). It appears at about the third hour after infection and its presence marks the end of the primary phase and the beginning of the secondary phase.

In the presence of guanidine, RNA replicase is not formed. Yet once the enzyme has been synthesized in the absence of guanidine it can perform its task in the presence of guanidine (Baltimore *et al.* 1963). Guanidine therefore blocks the formation of the viral RNA replicase but does not inhibit its activity.

Instead of studying the effect of guanidine on the formation of RNA replicase we may examine its action on the production of virions. Guanidine is added or removed at different phases of the viral cycle and the kinetics of the formation of virions is followed. If guanidine is added immediately after infection, viral development is blocked. If it is added at 1, 2 or 3 hr., viral development takes place (Fig. 1). The later guanidine is added the better the development (Lwoff & Lwoff, 1963, and unpublished work).

Thus guanidine blocks neither the replication of viral nucleic acid, nor the synthesis of the proteins of the capsid, nor the morphogenesis of the virion. Guanidine blocks only the formation of RNA replicase and does not interfere with the events of the secondary phase. Biological and biochemical data are in agreement and complement each other.

If guanidine is added at zero time and removed after 2 or 3 hr., the length of the latent phase is increased and the development slower. Moreover, the presence of guanidine from 0 to 1 hr. has little or no effect on the kinetics of viral development. But its presence from 1 to 2 hr. or from 2 to 3 hr. increases the latent period by about only 1 hr.

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Fig. 1. Effect of guanidine on the development of the guanidine-sensitive poliovirus (A. Lwoff & M. Lwoff, unpublished work). Contrary to all other experiments this one was performed with the heat-resistant mutant (critical temperature 40.8°) and at 39° . Guanidine (final concn. 1mM) was added at: $\times$, 0 hr.; $\blacksquare$, 1 hr.; $\triangle$, 2 hr.; $\bullet$, 3 hr. No guanidine was added in the control experiment ($\circ$).

(Fig. 2). Thus the presence of guanidine during the primary phase delays the formation of RNA replicase.

Inhibitors possessing an amino radical

The inhibitory action of guanidine on the development of poliovirus was discovered by Richtsel *et al.* (1961), but the search for substances possessing an antiviral activity had begun many years before. Already in 1949 the inhibitory effect of some amidines had been discovered (Rafelson, Pearson & Wintzler, 1950; Eaton, Levenson & Perry, 1952; McClelland & Van Rooyen, 1949) and 2 years later that of thiosemicarbazones (Hamre, Brownlee & Donovan, 1951; Thompson, Price & Minton, 1951). Thereafter it was found that a number of normal metabolites also block viral multiplication. This is the case for L-arginine, DL-lysine, DL-ornithine, L-histidine (Eaton, Magasanik, Perry & Karibian, 1951; Rafelson *et al.* 1950), tryptophan (Rafelson *et al.* 1950), glycine (Rappaport, 1956), phenylserine (Pons & Preston, 1961), guanidinoacetic acid (Eaton *et al.* 1951), glutamine (Eaton & Scala, 1961) and ammonia (Eaton & Scala, 1961; Jensen & Lin, 1961; Jensen, Force & Unger, 1961). The inhibitory action of these metabolites is observed only for concentrations ranging from 2 to 10mM.

Fig. 2. Effect of the addition of guanidine at various phases of the life cycle on the development of the guanidine-sensitive poliovirus (Lwoff & Lwoff, 1963). The medium used was lactalbumin hydrolysate-yeast extract supplemented with 2% of ox-embryo extract; the temperature was 36° . In (a), guanidine (final concn. 1mM) was added at: $\blacksquare$, 0 hr.; $\circ$, 1 hr.; $\triangle$, 2 hr.; $\times$, 3 hr. In (b) and (c) guanidine (final concn. 1mM) was present from: $\times$, 0-1 hr.; $\triangle$, 0-2 hr.; $\circ$, 0-3 hr.; $\blacksquare$, 0-8 hr. In (d), guanidine (final concn. 1mM) was present from: $\times$, 0-1 hr.; ∇ , 1-3 hr.; $\triangle$, 2-3 hr.; $\circ$, 0-3 hr. In each case no guanidine was added in the control experiment ($\bullet$).

All these substances possess an amino group, which is the only structure they have in common. It is therefore not surprising that this tenuous chemical parenthood did not suggest to someone a common mechanism of action. Yet this is the case at least for many of them, as will be shown by the study of the guanidine-resistant variant that has to be introduced here.

If the original sensitive strain of poliovirus is seeded in the presence of 1mM-guanidine, the cells are protected from infection. Yet the protection lasts only a few days and the cells are finally lysed. The death is caused by the multiplication of a guanidine-resistant variant of the virus. The development of the resistant strain is not influenced

at all by 1 or 2 mM-guanidine. The action of various inhibitors on the guanidine-resistant mutant was investigated. It turned out that it is resistant not only to guanidine but also to a number of substances, e.g. L-arginine, L-ornithine or glycine (Table 1).

Table 1. *Comparative effect of various inhibitors on the development of the original guanidine-sensitive strain of poliovirus and of a mutant resistant to 1 mM-guanidine*

The figures represent the percentage yield in a one-step growth cycle as compared with the control without inhibitor (Lwoff, Lwoff & Koch, 1963a; A. Lwoff & M. Lwoff, unpublished work).

Inhibitor	Concn. of inhibitor (mM)	Percentage yield	
		Original strain	Guanidine-resistant strain
L-Arginine	25	10	102
	5	16	110
Ornithine	20	23	106
Citrulline	20	30	85
Glycine	20	30	106
Carbazide	2	52	100
	10	25	115
	2	30	
(NH ₄) ₂ SO ₄	0.5	45	
	20	13	
	10	48	133
Amidinourea	20	0.3	18
Creatine	50	30	87
Uracil	4	32	38
Isatin β -thiosemi-carbazone	0.02	12	54
	0.04	7	43
	0.08	0.6	26

Moreover, its sensitivity towards isatin β -thiosemi-carbazone, carbazide, thiourea, thiosemicarbazide, amidinourea and ammonia is greatly decreased (Lwoff & Lwoff, 1964b). Thus the mutation conferring the resistance to guanidine suppresses or decreases the sensitivity towards a variety of substances possessing an amino group. These substances have therefore a common mode of action.

A few additional data have now to be considered: (1) Glutamine, semicarbazide and agmatine do not inhibit the guanidine-sensitive strain (Table 2). Thus not all substances possessing an amino group are inhibitory (Lwoff & Lwoff, 1964b). (2) Urea is inhibitory. Yet the guanidine-resistant mutant is as sensitive to urea as the original strain. Moreover, a mutant resistant towards urea (0.2 M) has been obtained, but it is nevertheless sensitive to guanidine. Thus the sensitivities to guanidine and to urea are controlled by different mutations (Lwoff, Lwoff & Koch, 1963a).

To interpret these data two hypotheses may be considered: (a) The inhibitory substances possessing an amino group act directly, by themselves, on viral development. (b) The inhibitory substances are metabolized to produce an excess of substances with an amidino group which are responsible for the inhibition. This hypothesis (b) is in agreement with our knowledge of the arginine cycle. Ammonia, or any substances liable to be deaminated, will set the Krebs cycle in motion. As a result an increased concentration of compounds with an amidino or ureido group is likely to occur. Among them are not only arginine and citrulline but also glycyamine and creatine. This hypothesis is considered again below when the problem of the guanidine-exacting strain is discussed. Whatever the case might be, the guanidine-resistant mutant is

Table 2. *Effect of various substances on the development of the original guanidine-sensitive strain of poliovirus and of two of its mutants*

Sensitivity: The substance is inhibitory (+), or not (0). $\pm$, The inhibitory effect is smaller than for the original strain. Activity: The substance replaces guanidine (+), or not (0), in the development of the guanidine-exacting strain. The results were obtained by A. Lwoff & M. Lwoff (unpublished work).

	Sensitivity		Activity
	Original guanidine-sensitive strain	Guanidine-resistant strain	Guanidine-exacting strain
Glutamine, semicarbazide, agmatine	0	0	+
Guanidine, 1-methylguanidine, 1,1-dimethylguanidine, L-arginine, L-ornithine, glycine, carbazide, ammonium salts	+	0	+
Creatine, amidinourea, thiourea, thiosemicarbazide, uracil, isatin β -thiosemicarbazone	+	$\pm$	+
Urea	+	+	0

resistant towards a number of substances possessing an amino group.

The antiguanidines

The development of the poliovirus is, in general, completely inhibited by 1mm-guanidine. Sometimes, however, a slight development is observed. With smaller concentrations of guanidine, e.g. 0.3mm, the inhibition varies, depending on the experiment, from 10 to 60%. Our first experiments were performed in a rather complex medium containing lactalbumin hydrolysate and yeast extract. It was suspected that this medium was responsible for the variations observed. The lactalbumin-yeast medium was replaced by Earle's medium (a solution of salts containing glucose), which was supplemented with ox-embryo extract. In such a medium, 0.3mm-guanidine inhibits viral development completely.

If Earle's medium is supplemented with yeast extract, the inhibitory effect of 0.3mm-guanidine is greatly decreased and the effect of 0.2mm-guanidine suppressed. Thus something present in yeast possesses an antiguanidine activity (Fig. 3). To identify the responsible substance one just had to fractionate yeast and test the fractions for their antiguanidine activity.

In 1936, while working in the Molteno Institute under the guidance of David Keilin, we had to fractionate baker's yeast to identify the growth

factor V (vitamin) necessary for *Haemophilus influenzae*. This was done in the Department of Biochemistry, in the students' laboratory that Sir Frederick Gowland Hopkins had kindly put at our disposal. In all, 30lb. of yeast was treated. This was a terrible mess. It finally ended up with a few milligrams of coenzyme I and II, which proved to be the growth factor V, and so we developed a great 'love' for yeast. In 1964, we had to fractionate yeast once more. At this point of the evolution of the work an important discovery was made. Since 1936 yeast has lost an important quality: it is no longer conducive to fractionation. Yeast has obviously aged. This is not surprising after an interval of 28 years.

So we decided that instead of fractionating yeast it would be much wiser to perform shelf experiments and test the antiguanidine activity of yeast components that were already known. In the meantime it was found that Eagle's medium had antiguanidine properties. (A medium containing the vitamins and amino acids at four times the original concentration was utilized.) Eagle's medium contains the vitamins of the B group, a mixture of amino acids and choline. The vitamins proved to be inactive. The amino acid mixture was active. Two amino acids are mainly responsible for this activity, namely L-methionine and L-valine (Fig. 4). Choline proved active too (Lwoff &

Fig. 3. Antiguanidine effect of yeast extract on the development of the guanidine-sensitive poliovirus (A. Lwoff & M. Lwoff, unpublished work). The medium used was Earle's medium supplemented with 2% of ox-embryo extract. The Figure shows the percentage yield in virus plaque-forming units after one cycle of development (8 hr.). Yeast extract (▲, 0.1%; ▽, 0.3%) suppresses or decreases the inhibitory effect of guanidine. No yeast extract was added in the three control experiments (x, o, ●).

Fig. 4. Effect of the antiguanidines on the development of the original guanidine-sensitive strain in Earle's medium supplemented with 2% of ox-embryo extract (Lwoff & Lwoff, 1964d). In (a), the additions (final concentrations given) at 0 hr. were: x, guanidine (0.3mm); ●, guanidine (0.3mm) + choline (0.033mm); ▽, guanidine (0.3mm) + L-valine (1.5mm). In (b), the additions (final concentrations given) at 0 hr. were: x, guanidine (0.3mm); □, L-methionine (0.4mm); ■, guanidine (0.3mm) + L-methionine (0.4mm). In each case no guanidine was added in the control experiment (○).

Lwoff, 1964d). L-Alanine (4mM) and L-leucine (1.5mM) have a slight antiguanidine activity, but only in the presence of the vitamins of the B group. With alanine, one of the active vitamins is folic acid. Moreover, yeast extract and the reinforced Eagle's medium are each more active than choline, methionine and valine, whether the amino acids are isolated or added together. For the time being, one can say that a number of compounds are antiguanidine and that the antiguanidine activity of yeast extract and of Eagle's medium is due to the action, and maybe the interplay, of numerous substances.

How could the antiguanidine act? The inhibitory activity of methylguanidine and of 1,1-dimethylguanidine is much smaller than that of guanidine. Methionine and choline are methylating agents. The antiguanidines could therefore act by methylating guanidine. To test this hypothesis cells were incubated for 3 hr. in the presence of labelled guanidine and of choline or methionine. If only one ten-thousandth of the guanidine had been methylated an additional spot would have been detected by chromatography. This was not the case: the experiments were entirely negative, whether the cells were normal or infected. It is therefore unlikely that antiguanidines act by transforming guanidine into an inactive substance. Another hypothesis is that antiguanidines act by competing with guanidine.

To test this latter hypothesis, the concentration of guanidine was doubled (0.6 instead of 0.3mM) and the concentration of choline and of methionine was increased 2-, 4- and 8-fold. No antagonistic effect was observed. Under experimental conditions, the antagonism with methionine and choline is obviously not of the competitive type. There is, however, a slight indication of a competitive-like action in yeast, at least when the guanidine concentration is below 0.5mM. The fact that methionine and choline are devoid of antiguanidine activity when the concentration of guanidine is 0.6mM is difficult to interpret. A hypothesis is nevertheless proposed below.

When the guanidine-sensitive strain of poliovirus is subcultured in the presence of 0.5mM-guanidine, resistant mutants are selected. They are not inhibited by 0.5mM-guanidine, but are inhibited by 1mM-guanidine. It is therefore possible that the virus possesses a number of sites with different affinities for guanidine. The antiguanidines would alter the receptivity of some of the receptor sites only. Antiguanidines could act by producing an allosteric transition, decreasing the affinity of the viral protein for guanidine. The antiguanidines were discovered in April 1964, and this accounts for the scarcity of our data. A systematic study will be necessary to know how they act.

Some effectors of viral development

Guanidine is a specific inhibitor of viral development, but it is not the only one. Before a hypothesis on the mechanism of its action is suggested some other inhibitors are briefly discussed.

Supraoptimum temperatures. The development of poliovirus is temperature-dependent (Fig. 5). The production of virions is optimum in a narrow temperature range and inhibited above and below. Supraoptimum temperatures block an event that takes place at a given phase of the life cycle and

Fig. 5. Effect of temperature on the development of the guanidine-exacting poliovirus (A. Lwoff & M. Lwoff, unpublished work). The infected cells were maintained in the lactalbumin hydrolysate-yeast extract medium supplemented with 2% of ox-embryo extract without guanidine for 3 hr. at 36°. The flasks were then transferred to water baths at different temperatures: ●, 36°; ○, 38.5°; ×, 38.6°; ■, 38.7°; □, 38.8°; ▲, 38.9°; △, 39°. After 3 min., when temperature equilibrium had been reached, guanidine was added (final concn. 1mM) and the viral development followed. The increase of temperature has three effects: (a) the period between the addition of guanidine and the onset of viral development is increased; (b) the rate of viral development is decreased; (c) the yield is decreased (see Fig. 6).

Fig. 6. Influence of temperature on the development of the guanidine-exacting poliovirus (A. Lwoff & M. Lwoff, unpublished work). The yield was plotted against temperature. O, Experiment represented in Fig. 5; ∇ , another experiment of the same type. In the critical range of temperature an increase of 0.1° decreases the yield by a factor 2.

may be completed within a few minutes. It is the sensitive critical event (Lwoff, Roizman & Lwoff, 1962) that marks the transition between the latent primary phase and the secondary phase of the cycle. It controls the replication of the viral RNA and we believe that it affects the formation of the RNA replicase.

Within a critical zone of temperature, an increase of 0.1° decreases the viral yield by a factor two (A. Lwoff & M. Lwoff, unpublished work) (Fig. 6). No other phenomenon seems to be known that exhibits such an exquisite temperature-sensitivity. The physical chemists I have consulted have proposed two hypotheses: (a) a very small number of bonds is involved, perhaps only one; (b) a relatively large number of identical bonds is involved.

Let me add that the effects of supraoptimum temperatures are reinforced by urea. They are counteracted by heavy water. So hydrogen bonds are perhaps involved. Whatever the case might be, by growing the poliovirus at supraoptimum temperatures, thermoresistant mutants can be selected. Thus thermosensitivity is controlled by the genetic constitution of the virus; the action of supraoptimum temperatures is not mediated by the cell. Supraoptimum temperature is a specific viral inhibitor.

Infraoptimum temperatures. Supraoptimum temperatures do not inhibit the events of the latent

phase. The events, however, are considerably delayed or even blocked by infraoptimum temperatures. Moreover, the effects of infraoptimum temperatures are reinforced by heavy water. From psychrosensitive (i.e. cold-sensitive) strains, psychrosensitive mutants can be selected. Thus psychrosensitivity as well as thermosensitivity is controlled by the genetic material of the virus and not by the cell, and cold is a specific viral inhibitor.

It happens that those mutations that decrease the thermoresistance increase psychrosensitivity and vice versa. The psychrosensitivity of the latent phase and the thermosensitivity of the critical event are therefore controlled by the same structural gene.

Heavy water. At optimum and infraoptimum temperatures, heavy water inhibits the development of the poliovirus. Heavy water reinforces the effect of infraoptimum temperatures, and decreases the effect of supraoptimum temperatures. At a concentration of 50%, heavy water decreases the apparent temperature by $3-4^\circ$. We do not know whether the effects of heavy water are primary or secondary. However, it was a surprise to find that some mutants of poliovirus are insensitive to heavy water. This is the case for one of our guanidine-resistant mutants; another proved to be sensitive. It is true also for our guanidine-exacting strain and for one urea-resistant strain (Lwoff & Lwoff, 1964c).

The kinetics of viral development of the D_2O -resistant mutants is the same at 33° , 36° and 38.5° in water and in the presence of 50% D_2O . This means that heavy water neither increases the length of the latent period at infraoptimum or optimum temperatures, nor decreases the effect of supraoptimum temperature. Here again, as with the mutations controlling thermo- or psychrosensitivity, an alteration of the physiology of the latent phase is connected with an alteration of the physiology of the critical event. Things happen as if the effects of heavy water, during the latent phase and during the critical event, depend on one and the same structure. The mutations that alter the physiology of the latent phase also alter the physiology of the critical event. It is therefore clear that the effects of heavy water depend on a critical viral structure liable to be altered by a mutation; D_2O is a specific viral effector.

Discussion. One hypothesis accounts for all the data on the action of temperature and heavy water (Lwoff & Lwoff, 1961; Lwoff, 1962). During the latent phase of the development of poliovirus, one step in the formation of the RNA replicase is counteracted by heavy water and by infraoptimum temperatures. This step is thermoresistant. During the critical phase another step is counteracted by supraoptimum temperatures, the effect of which

is decreased by heavy water. This step is thermosensitive. The comparative developmental physiology of hot and cold strains leads to the conclusion that both steps are determined by the same structural gene. Before discussing the nature of this step we have to come back to guanidine and to specific inhibition of viral development.

The specific inhibition

Guanidine inhibits viral development by preventing the formation of the viral RNA replicase. One could be satisfied with this conclusion. However, one has to ask another question: what is the mechanism by which guanidine inhibits the formation of a specific viral enzyme?

Under experimental conditions guanidine does not visibly affect cellular growth and multiplication. Mutants insensitive to the inhibitory effect of guanidine and other specific inhibitors can be selected. This would not be true if the inhibitors blocked viral development by interfering with the cellular metabolism. Thus the inhibitors have to exert their action on the virus by interfering with a viral structure. The problem posed here is that of the mode of action of specific viral effectors.

Viruses are reproduced from their genetic material, which introduces into the host cell the information for the synthesis of viral molecules. The host cell provides the building blocks necessary for the synthesis of viral proteins and viral nucleic acid, i.e. the amino acids and the nucleotides. It provides also the ribosomes, the enzymes operative in the synthesis of proteins, the transfer RNA and energy in a suitable form. The development of a virus consists essentially in the transcription of the information (i.e. the replication of the genetic material), the translation of information (i.e. the synthesis of proteins) and, finally, the morphogenesis of the virion.

A number of agents are known that block viral multiplication. Among them are the inhibitors of the synthesis of nucleotides or amino acids, or of the production or utilization of energy-rich bonds. Among them are also analogues of nucleotides or of amino acids that are incorporated into the cellular nucleotides or into the proteins, thus giving rise to pathological structures unable to perform their normal functions. These types of agent block the development of viruses, either because they perturb the synthesis of the building blocks of viral molecules or because they are incorporated into viral molecules in place of normal metabolites. These agents that block viral development by interfering with cellular metabolism are not specific. We are interested in specific viral inhibitors.

The synthesis of essential metabolites necessary for the construction of viral molecules is mediated

by cellular enzymes. This process is therefore excluded as a possible target for specific antiviral agents. The translation of viral information, i.e. the synthesis of viral proteins on a viral RNA template, is also mediated by cellular enzymes. It is therefore unlikely that specific antiviral agents would interfere at this level. Could they act on the structure and functions of the nucleic acid itself?

A structural gene is a sequence of a few hundred nucleotides. A point mutation is the substitution of one nucleotide by another in the genetic material and, as a consequence, in the homologous messenger. It is difficult to visualize how a substitution of one nucleotide among many hundreds could modify the properties of the nucleic acid in such a way that a difference of 0.1° would markedly affect its structure or that it would become resistant towards guanidine. It seems therefore unlikely that specific antiviral agents act by modifying the structure of the viral nucleic acid.

The guanidine-sensitive strain of poliovirus multiplies in the presence of guanidine, provided that guanidine is added after the formation of the RNA replicase. Thus guanidine does not interfere with the replication of the viral RNA. The findings of Cords & Holland (1964) agree with these views. When cells are infected simultaneously with a guanidine-sensitive strain and a guanidine-resistant one, the RNA of the sensitive virus multiplies in the presence of guanidine, obviously thanks to the presence of the RNA replicase produced by the resistant helper virus. Cords & Holland (1964) conclude that guanidine does not act by modifying the RNA in such a way that it can or cannot replicate. The study of the kinetics of guanidine action had already led to the same conclusion. To interpret the action of supraoptimum temperatures, and that of guanidine and heavy water (Lwoff, 1962), we proposed the hypothesis that specific viral inhibitors act on viral proteins. A specific viral protein is known that controls the transcription of the viral nucleic acid, i.e. the duplication of the viral genetic material of RNA viruses, namely RNA replicase. Guanidine blocks the formation of the RNA replicase. Here again we have to ask: how? A digression on protein synthesis and on allostery is here necessary.

Protein synthesis and allostery

Protein synthesis. Let us first introduce the terminology proposed by Monod, Wyman & Changeux (1965). Many globular proteins and many enzymes are composed of a definite, relatively small, number of identical units. The globular protein is an 'oligomer' and the units associated in the oligomer are the 'protomers'. When dissociated, i.e. free, they are called 'monomers'.

The synthesis of a protein is a complex process. The first step is the organization of the individual amino acids in a specific sequence and in the correlative formation of peptidic bonds. This process is described either as 'sequentialization', 'patternization', or 'diataxy' ('to put in order'). Yet diataxy is only the first step of protein synthesis. The second is morphogenesis. For the catena has to undergo a series of alterations that confer on it the secondary, the tertiary and the quaternary structure. Diataxy and morphogenesis are often united under one and the same expression, namely 'protein formation' or 'protein synthesis'. Yet the nature of the two processes is essentially different.

The primary structure is controlled by the corresponding structural gene and messenger. The structures of higher order depend, of course, on this primary structure, but the situation is complicated by the fact that the quaternary structure is liable to be altered by a variety of agents. Here allostery has to be introduced.

Allostery. An enzyme possesses at least three categories of receptors: (a) the active receptor, i.e. the receptor for the substrate; (b) the receptor for specific inhibitors responsible for retro-inhibition; (c) the receptors of activation, i.e. for effectors, which increase the affinity of the enzyme for the substrate. There is, of course, no competitive interaction between substances acting on different receptors.

The sensitivity of an enzyme towards effectors is controlled by its anatomy. As a consequence of mutations of the structural gene, the structure of the protein may be modified in such a way that a given category of effectors becomes inactive. The same result is obtained by incorporation of amino acid analogues. Finally, agents, e.g. mercuric salts, that bind to the thiol or histidyl groups may suppress the sensitivity to the specific inhibitor or modify the activity of the enzyme. Thus the properties of an enzyme—its activity towards its substrate and its sensitivity to inhibitors or inactivating agents—can be modified by the binding of a particular ligand. The presence of a given ligand at a given site modifies the reactions of the protein to ligands attached on other distinct sites. The altered properties are due to reversible transitions of the structure of the protein. They are called 'allosteric transitions'.

By definition, allosteric interactions correspond to reversible transitions between different types of quaternary structures. They do not correspond to a transition between monomers and oligomers. However, it is known that the association of monomers into an oligomer is controlled by their structure. This is the case for the β -galactosidase of *Escherichia coli*. The enzyme is a tetramer. As a consequence of certain mutations of the cor-

responding structural gene abnormal monomers are produced that are unable to associate. Thus the quaternary structure is governed by the tertiary structure, which in turn is determined by the primary one. It is therefore theoretically possible that a reversible alteration of a monomer could modify the balance between the isolated and aggregated phase of the oligomer or even command the possibility of their association.

The specific inhibitors: mechanism of action

Let us now come back to viral development, and try to see if knowledge of protein synthesis is of any help in interpreting the data on the development of poliovirus. The conclusion was reached that specific antiviral agents (a) are unlikely to act directly on the structure and function of the viral nucleic acid, and (b) are unlikely to interfere with diataxy, i.e. with that step of the formation of the viral proteins which is mediated by cellular enzymes.

Guanidine blocks the 'formation' of the viral RNA replicase. If guanidine does not interfere with diataxy it necessarily interferes with morphogenesis. One hypothesis is that guanidine alters the structure of the monomer. As a consequence, either an abnormal, enzymically inactive, oligomer is produced, or the association of the monomer into an oligomer is made impossible. The result is the same, namely the absence of any enzyme activity (Scheme 1).

Baltimore *et al.* (1963) have reached another conclusion. Guanidine would (a) interfere with the messenger function of the RNA, i.e. with diataxy, and (b) perhaps inhibit most, if not all, virus-induced protein synthesis.

If hypothesis (a) is accepted, then hypothesis (b) is logical, for it is unlikely that guanidine or other effectors could affect the activity of one structural gene only. This view is in contradiction with the data on the kinetics of guanidine action in the guanidine-sensitive strain as well as in the guanidine-exacting strain, which will be considered now.

Scheme 1. Hypotheses on the mechanism of action of some specific effectors of poliovirus development.

The guanidine-exacting strain

Action of guanidine. When the guanidine-resistant strain is subcultured serially in the presence of guanidine, one ends up with an exacting strain (Loddo, Muntoni, Spanedda, Brotzu & Ferrari, 1963).

The exacting strain develops only in the presence of guanidine and not at all in its absence. In the absence of guanidine the viral RNA does not multiply. The absence of multiplication is due to the fact that the RNA replicase is not formed. It is synthesized, of course, when guanidine is present (Eggers, Reich & Tamm, 1963). Once the enzyme has been produced in the presence of guanidine it can work in its absence. Guanidine is therefore necessary for the formation of the enzyme and not for its activity (Eggers *et al.* 1963).

To know at what phase the guanidine acts it was added or removed at various phases of the cycle: (1) The infected cell is left without guanidine for 2–3 hr. and guanidine is then added. Almost instantly the formation of virions starts at the normal rate. After 5 min. the increase is already significant. These experiments show that guanidine is not necessary for the completion of the events of the latent period (Lwoff, Koch & Lwoff, 1963), and that the replication of the nucleic acid and the morphogenesis of the virion take place in less than 5 min.

Fig. 7. Effect of a 10 min. pulse of guanidine on the development of the exacting strain of poliovirus (A. Lwoff & M. Lwoff, unpublished work). The infected cells are suspended in the lactalbumin hydrolysate–yeast extract medium. In the control (Δ), guanidine (final concn. 1 mM) was added at 3 hr. and left throughout. In the other flasks guanidine was added respectively at: $\bullet$, 1 hr.; $\times$, 2 hr.; $\circ$, 3 hr.; then, after 10 min., in each case, the cell suspension was centrifuged (for 5 min.) and the cells were resuspended in a guanidine-free medium. The guanidine pulse is without effect at 1 or 2 hr. but effective at 3 hr.

(2) Guanidine is added at various intervals and removed after 10 min. by centrifugation of the cells and their resuspension in a guanidine-free medium. If the pulse is given at 0, 1 or 2 hr., the development is absent or negligible. But if guanidine is added from 3 hr. to 3 hr. 10 min., viral development takes place in the absence of guanidine. Thus guanidine is necessary for a short period, but it has to be present at the right time (Fig. 7).

Fig. 8. Effects of temperature and the guanidine pulse on the guanidine-exacting strain (A. Lwoff & M. Lwoff, unpublished work). Infected cells were suspended in the lactalbumin hydrolysate–yeast extract medium and maintained for 3 hr. at 36° in the absence of guanidine. The suspension was then subdivided into four flasks A, B, C and D. The contents of flask A were diluted 5-fold, and this diluted suspension was then diluted 6-fold: (a) in a medium containing guanidine (final concn. 1 mM) ($\bullet$); (b) in a medium containing guanidine (final concn. 0.033 mM) ($\square$). The contents of flask B were left at 36°, and those of flasks C and D were transferred respectively at 14° and 40°; then guanidine was added to the three flasks (final concn. 1 mM) after 3 min. A 30-fold dilution was performed respectively after 2 and 15 min. (final concn. of guanidine 0.033 mM) in media at the same temperature as that of the mother flask, i.e. 14°, 36° or 40°. Then 3 min. later the 14° and 40° flasks were transferred back to 36°. Thus the suspensions were exposed to a medium containing guanidine (final concn. 1 mM) (a) for 2 min. at 14° ($\blacksquare$), 36° ($\times$) or 40° ($\circ$), or (b) for 15 min. at 14° (Δ), 36° ($\blacktriangle$) or 40° (∇). No guanidine was added in the control experiment ($\blacktriangledown$). This experiment shows that a pulse of 15 min. at 36° has allowed the same development as in the control, whereas the development was slower and the yield smaller for 14° and 40°. A 2 min. pulse is still effective.

(3) The infected cells are left without guanidine for 3 hr. Guanidine is then added to give a concentration 1 mM. At intervals the infected cells are diluted in a guanidine-free medium. The final concentration is 30 or 33 μ M. The development takes place after a pulse of 15 min. Sometimes a 2 min. or a 1 min. pulse allows a yield of 50% (Figs. 8 and 9).

The fact that, after the addition of guanidine, viral development starts immediately at the maximal rate shows that guanidine penetrates rapidly into the cell. The fact that a short pulse allows viral development could mean that guanidine leaves the cell slowly.

Experiments with labelled guanidine have shown that the equilibrium of extracellular and intra-

cellular guanidine is reached in less than 1 min. at 0° as well as at 36°. The penetration and exit of guanidine across the membrane of the cell is a mere diffusion (C. Burstein, E. J. Batchelder & A. Lwoff, unpublished work).

Specificity. To what extent is the action of guanidine specific? The working hypothesis was that its action is due to an amidino or a ureido group. So a number of substances were tested, e.g. methylguanidine, carbazide and semicarbazide, citrulline, guanidinoacetic acid, creatine and creatinine, thiourea, thiosemicarbazide, uracil and isatin β -thiosemicarbazone. They all proved to be active. Finally, it seemed wise to test, as controls, substances devoid of an amidino or ureido radical. Ornithine was active, glycine also, as were glutamine, hydroxylamine and finally ammonia (Lwoff, Lwoff & Koch, 1963b).

As seen from Table 2, a number of substances possessing an amino group can replace guanidine for the development of the guanidine-exacting strain. One substance is inactive: urea. Urea is not metabolized by the mammalian cell and, as mentioned above, sensitivities to urea and to guanidine are controlled by different structures.

The maximal development is observed for concentration of guanidine 1 or 2 mM. At 5 mM the development is inhibited and the final yield decreased. At concentrations below 1 mM the latent phase is longer, the rate of multiplication slower and the yield smaller. Among the substances tested, guanidine gives the maximal yield. In the presence of other substances the yield varies from 1 to 90% of that of the guanidine control.

The concentration at which the various effectors act is subject to considerable variation (see Fig. 10).

Fig. 9. Influence of a guanidine pulse on the development of the guanidine-exacting poliovirus (A. Lwoff & M. Lwoff, unpublished work). Infected cells were suspended in the lactalbumin hydrolysate-yeast extract medium and maintained for 3 hr. in the absence of guanidine at 36°. The suspension is then subdivided in two flasks A and B. The contents of flask A were diluted 33-fold: (a) in a medium containing guanidine (final concn. 1 mM) (■); (b) in a medium containing guanidine (final concn. 0.03 mM) (▲); (c) in a medium containing no guanidine (control) (□). To the contents of flask B was added guanidine (final concn. 1 mM). The suspension was diluted 33-fold after 1 min. (×), 2 min. (○) and 10 min. (●), the final guanidine concn. therefore being 0.03 mM as in A(b). The final yield without guanidine is 0.8% of that in A(a) with guanidine (1 mM). It is 1.3% with guanidine (0.03 mM); for guanidine (1 mM) pulses of 1, 2 and 10 min. respectively it was 24, 24 and 30%.

Fig. 10. Development of the guanidine-exacting strain as a function of the concentration of glutamine, ammonium sulphate and guanidine (A. Lwoff & M. Lwoff, unpublished work). The medium used was lactalbumin hydrolysate-yeast extract supplemented with 2% of ox-embryo extract. The Figure shows the final yield at the end of one cycle (8 hr.) as compared with the development in the presence of guanidine (final concn. 1 mM), which gives the maximal yield (100%). The additions were: ○ and ×, L-glutamine; ■, ammonium sulphate; ▽, guanidine; ●, none (control).

Glutamine, for example, is still active at a concentration $0.1\mu\text{M}$. This might be a question of penetration into the cell. As mentioned above, the entrance and exit of guanidine into the K.B. cell is, under experimental conditions, a mere diffusion. The problem of the penetration of glutamine is not yet solved. A few experiments have shown, however, that the concentration of glutamine is higher in the cell than in the medium, and that the rate of penetration is temperature-dependent. Things happen as if the movement of glutamine would at least partly depend on the activity of an enzyme or, less specifically, on cellular metabolism (C. Burstein, E. J. Batchelder & A. Lwoff, unpublished work). The main problem, however, is that of the mode of action of the active substances. We have already discussed the inhibitory effect of compounds possessing an amino group on the development of the guanidine-sensitive strain. The hypotheses were proposed that these substances would set the Krebs arginine cycle in motion and increase the concentration of substances possessing a guanidino, amidino or ureido group. This hypothesis could apply to the effectors of the guanidine-exacting strain as well as to those of the sensitive strain. One objection can be raised to this view: glutamine, semicarbazide and agmatine, which replace guanidine for the exacting mutant, are devoid of inhibitory action on the sensitive strain. It is therefore possible that some of the substances tested act directly on the virus. The fate of semicarbazide in the animal cell is now being investigated.

Discussion. Let us now forget about specificity and discuss the problem of the mechanism of action of guanidine considered as an indispensable effector of the exacting strain.

The experiments on the kinetics of guanidine action mean that guanidine is necessary, for a short period only, provided that it acts at the right time. Guanidine probably acts on something that has to be produced during the primary phase. The most likely hypothesis is that this 'something' is the monomer of the RNA replicase (Scheme 2).

Scheme 2. Hypotheses on the mechanism of action of guanidine for the exacting poliovirus.

In the guanidine-exacting strain, guanidine would be necessary in order that either the monomers can associate or their structure be such that the oligomer is enzymically active.

The experimental data of Cords & Holland (1964) agree with these views. When cells are infected simultaneously with a guanidine-exacting and with a guanidine-independent strain of poliovirus, the RNA of the exacting strain multiplies in the absence of guanidine, obviously thanks to the RNA replicase produced by the guanidine-independent variant. Cords & Holland (1964) conclude that guanidine does not act by modifying the RNA in such a way that it cannot replicate but acts on viral proteins by influencing their synthesis, configuration and function. The authors do not specify the meaning of the word 'synthesis'. From the context it obviously does not mean configuration and therefore can only mean diataxy. On this point we disagree. If, however, configuration only is concerned, we are, of course, in complete agreement with this hypothesis, which we had proposed independently some time ago (Lwoff, 1962; Lwoff & Lwoff, 1963, 1964a; Lwoff, Lwoff & Koch, 1963a).

All that has been said about guanidine applies to 2-(α -hydroxybenzyl)benzimidazole (HBB), which also inhibits the formation of the RNA replicase of the HBB-sensitive strain and is necessary for its formation in the HBB-exacting strain (Eggers *et al.* 1963; Tamm & Eggers, 1963).

Remarks on exactness and on activation

Exactness. As mentioned above, when the guanidine-resistant strain is subcultured in the presence of guanidine, a guanidine-exacting mutant is selected. Obviously the mutant, in the presence of guanidine, possesses some selective advantage over its ancestor. It turns out that the latent period of the guanidine-exacting strain lasts about 2 hr., instead of 3 hr. for the guanidine-resistant one. This shortened latent period could account for the selection of the exacting mutant. Is the selection of the guanidine-exacting mutant the particular expression of a general phenomenon? Could it be, for example, that multiplication in the presence of a given category of substances would tend to select mutants for which this substance is an activator?

The urea-resistant mutant has been subcultured serially in the presence of 0.2M -urea. After 34 passages, 0.2M -urea increases the viral yield by 60% (A. Lwoff & M. Lwoff, unpublished work). The strain might be on the way to exactness. Whatever the case might be, for this strain, urea has become an activator.

Activation. Inhibitors of viral development have been described as well as antagonists of inhibitors. If inhibitors exist, why not activators too? After

all, inhibitors might be present in any medium and in any cell and, in the presence of an inhibitor, an anti-inhibitor is an activator.

Semicarbazide is devoid of antiguanidine activity but possesses an interesting property. Added to Earle's medium, it decreases the length of the latent phase, increases the rate of viral development and increases the yield of infectious particles. Among the numerous substances tested, semicarbazide is the only one that accelerates the development of poliovirus and, so far as we know, is the first example of a specific activator of viral development. (The enhancing activity of actinomycin on viral development is mediated by the host cell.) Things happen as if semicarbazide would favour the formation of the RNA replicase and perhaps increase its activity (Fig. 11).

Virologists are used to growing viruses in cells maintained in artificial media. A theoretical possibility exists that mutants are thereby selected for which constituents of the medium or products of cell metabolism have become activators. If the activator is an amino acid or a nucleotide that is a building block of the virus it has to be present so that the virus can develop. Its activity as effector would therefore be difficult to detect. Since glutamine and so many amino acids and cellular metabolites do replace guanidine for the exacting

strain one is surprised that the requirement for guanidine was discovered. Obviously, in studying viral development, great attention should be given to the constitution of the medium and to the concentration of its components. Virologists should be aware of the fact that a cell contains activators, inhibitors and anti-inhibitors, and that viral development is the result of the interplay of numerous factors of which only a few are known.

Conclusions

The regulation of bacterial functions is controlled by food and by metabolism. The development of an animal is controlled by those cell-made substances called hormones. The experimental data presented above show that the development of the poliovirus is also controlled by a variety of cellular metabolites, which act as activators, inhibitors or anti-inhibitors.

The mechanism of action of the effectors of viral development has been discussed. To account for all the experimental data a coherent unifying concept has been proposed. The specific effectors are unlikely to interfere with diataxy, i.e. with the assemblage of amino acids into a polypeptidic chain. They could act by altering the morphogenesis of viral enzymes or proteins in such a way that the formation of the right tertiary or quaternary structure is prevented.

The evolution of viral proteins, like the evolution of proteins in general, has thus led to the development of specific sites able to accept specific effectors. These effectors control the morphogenesis, structure and consequently the activity of viral proteins, i.e. in last analysis the viral life cycle itself.

The sensitive structures are certainly not present to allow virologists to 'play' with the virus, to enhance or inhibit its development or dream of a specific antiviral chemotherapy. The sensitive structures should confer some advantage to the virus. One of our tasks is to understand their meaning. The problem which has been posed in this Lecture, and not solved, is that of the role of cellular metabolites as effectors in the developmental physiology and evolution of viruses.

Appendix: Remarks on repressors of viral development

In a lysogenic bacterium the autonomous multiplication of the nucleic acid of the bacteriophage is blocked by a specific repressor. Induction of the phage development seems to be due to the production of a substance, perhaps in certain cases a derivative of adenine, which inactivates a repressor (Goldthwait & Jacob, 1964). Repressors of the development of animal viruses have not yet been

Fig. 11. Effect of semicarbazide (20 mM) on the development of the original guanidine-sensitive poliovirus (A. Lwoff & M. Lwoff, unpublished work). The medium used was Earle's medium supplemented with 2% of ox-embryo extract. The doubling time during the exponential phase is 13 min. for the control (●) and 9.6 min. in the presence of semicarbazide (O).

detected. However, the hypothesis should be considered according to which guanidine would turn an inactive aporepressor into a repressor, or vice versa, depending on the sensitivity or requirement of the strain. This repressor would have to control the activity of the structural gene corresponding to the RNA replicase. Three main objections can be raised against this hypothesis: (1) it is not in agreement with the data on the kinetics of the action of guanidine; (2) it is complicated; (3) I do not like it at all.

Yet, as long as we do not know how specific effectors of viral development act, both hypotheses should be borne in mind: action on the genetic material mediated by a repressor, direct action on a viral protein. Three examples will be given:

(a) Infection with the herpes virus is often latent. Something blocks viral development. The multiplication is induced by a variety of agents. One might conceive that, as a result of the altered cellular metabolism, a substance that is an effector of viral development either appears or disappears. It could, of course, act on a repressor as well as on a viral protein.

(b) The RNA oncogenic viruses multiply in the 'transformed' host cell without killing it. Something obviously controls viral multiplication and some sort of control mechanism has to be envisaged.

Here again the alteration of cellular metabolism induced by the virus could alter the concentration of a cellular metabolite acting as an effector of viral development and controlling its rate and extent just as the concentration of guanidine controls the rate and extent of the development of poliovirus. Here again the effector could as well alter a repressor as act directly on a viral protein.

(c) In many plants, the virus does not kill the infected cells and its multiplication stops. Obviously some altered condition interferes with viral development and here again both types of action, direct or indirect, can be envisaged.

Note added in proof. The study of the effect of antiguanidines on the development of the guanidine-exacting strain has revealed that choline and L-methionine decrease the viral yield at infraoptimum or optimum concentrations of guanidine (not more than 1 mM). Moreover, at supraoptimum, inhibitory, concentrations of guanidine (2–4 mM) the methionine and choline increased the yield. The hypothesis has been proposed that, in the guanidine-sensitive strain, guanidine would interfere with an essential methylation of a viral structure. In the guanidine-exacting strain, antiguanidines would interfere with an essential amidination (Lwoff & Lwoff, 1965).

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