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Cell-Mediated Immunity and the Major Histocompatibility Complex*

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Contents

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Prolog

Immunology today offers a sharp focal point for a number of general questions that confront biologists, biochemists, physiologists, and many others who are concerned with cellular interactions. In immunology, cellular immunology in particular, the immune *system* is the central concept. Although much progress has been made in documenting the constituents and many of the phenomena exhibited by the immune system, there is an urgent need to understand how the constituents interact to produce the phenomena. Though potentially misleading, a useful analogy is to view immunology as being similar to the lactose system, or phage system, of *Escherichia coli* in the pre-Operon days: We are looking for that conceptual framework which will solve the apparent conflicts and subsume the mass of amorphous data into a unified system in the same way that the Operon theory did. Much the same as *E. coli* has become the prototype organism for molecular biology, so the mouse is becoming the prototype organism for immunology.

Two principal areas of immunologic interest have provided the richest sources of information for probing the underlying principles of control and regulation of the immune system. One major area, which will not be treated in detail here, has been the arrangement of structural genes for immunoglobulin proteins and the manner in which their enormous repertoire of specific antigen-binding sites is generated. The interested reader can find several excellent discussions of this subject (*Hood* and *Prahl*, 1971; *Gally* and *Edelman,* 1972; *Hood* et al., 1975). The other area of intense activity is the cluster of genes in the region of the species-major histocompatibility gene complex. In the mouse this is called the histocompatibility-2, or H-2, complex. There are few aspects of immunobiology which are not related, either directly or indirectly, to this gene complex. The appearance of a new scholarly monograph, the *Biology of the Mouse Histocompatibility-2 Complex* (*Klein,* 1975), which gives a balanced and detailed appraisal of this key area, is both timely and welcome. However, the rate of advance in knowledge in this exciting area has been so rapid of late that the phenomenon of H-2 restriction remained inconceivable at the time Klein's monograph was compiled. The starting point for this essay is the phenomenon of H-2 restriction.

Probably the most important new advance to emerge in the past 10 years is that of H-2 restriction, and though apparently simple enough on the surface, the broader implications strike to the core of our essential beliefs of how an immune system operates. What, then, is H-2 restriction in general terms, and why is it being given such importance? To begin with, the immune system has two major classes of effector reaction, one based on immunoglobulins, or humoral immunity, the other based on cytotoxic cells, or cellular immunity. In essence, H-2 restriction is the constraint imposed on immune cytotoxic cells which requires that antigens determined by the murine H-2K and

H-2D genes be specifically recognized in order for the cytotoxic, or killer, effector function to be expressed. For example, a mouse immune to mouse pox (ectromelia) virus has cytotoxic cells that can kill virus-infected target cells, but only if the cytotoxic immune cell with specific antiviral recognition has specific anti-H-2 recognition in addition, and the H-2 antigen recognized must be "self" H-2. Following this idea of self H-2 recognition, questions arise such as why self and why H-2 self. When considered in conjunction with antiviral (nonself) specificity, as expressed in the cytolytic killer effector function, there are ramifications which reach beyond current concepts in immunology.

In this review I wish to introduce a new way of approaching problems in biologic systems by means of a detailed analysis of the H-2 restriction phenomenon and thereby show how new perspectives can lead to new avenues of experimentation. The review is divided into three parts: 1. Background and Current Concepts, which will orient newcomers to the field; 2. A Detailed Mechanism for H-2 Restricted Cellular Cytotoxicity, which defines the rules necessary to accommodate the present data; and 3. The Evolution of a Cell-Mediated Immune System, which constitutes an approach to testing the feasibility of the mechanism and consequences of H-2 restricted cytotoxicity developed in part 2.

1. Background and Current Concepts

The enigmatic phenomenon of H-2 restriction is at present a subject of intense investigation and is the point I wish to bring into focus here. There are several excellent reviews which summarize the experimental findings of a number of laboratories and which offer particular interpretations of the data (*Doherty* et al., 1976a,b; *Zinkernagel* and *Doherty*, 1976; *Lennox*, 1975; *Blanden* et al, 1976; *Bevan*, 1976). This essay is based on the murine H-2 system which I consider to be a prototype extensible in principle to all other species showing restricted activity of cytotoxic effector T cells. Consequently, it would be best to clarify the nomenclature of the murine major histocompatibility complex and make some working definitions.

1.1. Synopsis of H-2

The H-2 gene complex has been extensively analyzed in classical genetic terms and by detailed serology. In this section I have drawn from a variety of sources (*Klein*, 1975; *Klein* and *Shreffler*, 1971; *Shreffler* and *David*, 1975; *Murphy* and *Shreffler*, 1975) the essential information relevant to an understanding of H-2 restriction. The mouse is a diploid organism and thus carries two chromosomes with parallel sets of genes on each pair, one chromosome being maternally derived, the other paternally derived. In the studies to be discussed here, inbred or homozygous mice are used as the starting materials. For the sake of clarity, we can begin by considering only one chromosome or, as it is often called, one haplotype (as in haploid bacteria). On the 17th chromosome, or IX linkage group of the mouse, lies the H-2 gene complex, as illustrated in Figure 1. There are two loci of primary importance in H-2 restriction, which are termed H-2K and H-2D. These two loci, which are about 0.5 recombination units apart, control antigenic specificities found on two glycoproteins of mol.–wt. 45,000 that are present on virtually all cell types found in the mouse. The H-2K and H-2D glycoproteins have very similar structure and function, but they are antigenically distinct. It is not possible to state with certainty that the 45,000 dalton glycoproteins (termed here the H-2K and H-2D molecules) are the sole products of the H-2K and H-2D genetic regions, to be referred to as H-2K and H-2D genes. However, in the interest of clarity and simplicity, I will assume that H-2K and H-2D genes give rise to H-2K and H-2D molecules only, i.e., there is no other product coded in the genetic regions essential for H-2K and H-2D function.

One of the most outstanding features of these two genes is the very large number of allelic forms found in the mouse population at large, i.e., the loci are highly polymorphic. Allelic forms of H-2K and H-2D are identified by antigenic differences, and, as might be expected, there is considerable overlap in antigenic specificities between alleles of H-2K and H-2D, as well as specificities that are shared by H-2K and H-2D. As a first step in establishing a sense of order among this vast array of antigens, the more generally shared antigenic determinants were termed "public," and unique antigens were termed "private" to identify a particular allele of the gene. The antigenic composition of a particular allele will thus contain both public and private antigens; this entire grouping is denoted by a superscript letter symbol such as H-2K^{b}, H-2K^{d}, H-2K^{k}, H-2D^{d}, H-2D^{k}, etc. However, H-2K and H-2D genes have been isolated by pairs during the establishment of inbred mouse

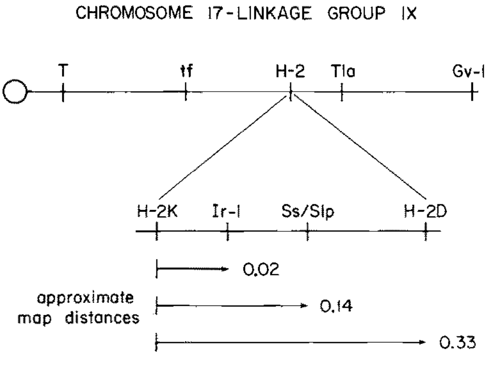


Fig. 1. H-2 genetic complex

strains and so occur as pairs, such as H-2K^k-H-2D^k, or H-2K^b-H-2D^b; these so-called haplotypes are given the shorthand symbol H-2^k and H-2^b. Since the H-2K and H-2D loci are separate, though closely linked, it is possible to obtain recombinant haplotypes from heterozygotes, such as H-2K^k-H2D^d, and so on.

Throughout this chapter frequent reference will be made to the use of H-2 congenic mouse strains (see *Klein,* 1975, for details). *Congenic* denotes that two strains of mice differ only in the H-2 genetic region; at all other loci on all other chromosomes, the H-2 congenic mice are assumed to be identical. There is a form of shorthand nomenclature in which the background strain genes are denoted by a letter/number of the strain code, then a period, and the code for the mouse strain from which the H-2 gene region was derived. For example, the congenic strain C.B is a Balb/c mouse which has the H-2 gene complex derived from B (or B6); the H-2 congenic strain B10.D2 is a C57B1/10 mouse which has the H-2 gene complex derived from DBA/2. Wherever possible the H-2 allele will be given in addition to the strain codes, e.g., B10 (H-2^b) or B10.D2 (H-2^d).

1.2. H-2 Restriction

1.2.1. Historical

The phenomenon of H-2 restriction applies to the effect of H-2K and H-2D genes, which via their molecular products behave as antigenic constraints in the effector reaction of killer T lymphocytes. The killer property of thymus-derived (T-) lymphocytes is expressed in vitro by their capacity to cause cytolysis of appropriate living target cells upon intimate contact of the killer with its target. It is in the analysis of what distinguishes appropriate form inappropriate target cells that we encounter H-2 restriction as an additional component to simple antigenic specificity. The situation is best illustrated by the use of detailed examples.

For a number of years, the only way to experimentally produce efficient killer T cells was to immunize against the major histocompatibility or H-2 antigens, i.e., the H-2K and H-2D alloantigens of the mouse were appropriate cell-surface antigens to which killer T cells could direct their activity (*Cerottini* and *Brunner,* 1974). All activity was directed at antigens determined by H-2K and H-2D genes, and by the use of H-2 recombinant targets, the activity was shown to be restricted to "private" specificities of H-2K and H-2D (*Brondz* et al., 1975). For example, the injection of Balb/c (H-2^d) cells into a C57B1/10 (H-2^b) mouse induces the formation of killer T cells which attack any target cell carrying either H-2K^d or H-2D^d antigens, irrespective of the other target cell-surface antigens.

Then came the startling observation that specific cytotoxic T cells generated during recovery from viral infection could kill target cells infected with the same virus only if there was H-2 matching between the killer and the target (*Zinkernagel* and *Doherty,* 1974a; *Doherty* and *Zinkernagel,* 1976; *Blanden* et al., 1975; *Zinkernagel* and *Dohery,* 1975; *Koszinowski* and *Ertl,* 1975: *Gardner* et al., 1975; *Lewandowski* et al., 1976). In other words, when killer T cells were generated with specificity for non-H-2 antigens (e.g., viral), there was a second component of specificity determined by H-2 antigens. The initial experiments with viral infections and H-2 restricted T-killing clarified previous similar observations made using trinitrophenylated lymphocytes as antigens and targets (*Shearer,* 1974). There followed an extension of the phenomenon to include non-H-2 (minor histocompatibility) antigens (*Gordon* et al., 1975; *Bevan,* 1975). Experimentally, *Zinkernagel* and *Doherty* (1974a) found that CBA mice (H-2^k) during recovery from LCM infection, had cells in their spleens which killed LCM-infected, but not uninfected H-2^k cells; whereas neither infected nor uninfected H-2^d cells were killed by immune CBA spleen cells. There was nothing special about the H-2^d target cells since Balb/c (H-2^d) mouse spleen cells, taken from an animal convalescing after LCM-infection, killed the LCM-infected, but not the uninfected, H-2^d target cells; neither infected nor uninfected H-2^k targets were killed. Specificity for viral antigens was demonstrated by reciprocal infections with LCM and ectromelia viruses (*Doherty* and *Zinkernagel,* 1976), and in each case of antiviral specificity there was in addition the requirement for H-2-matching between killer and target cells. Implicitly, there is H-2-matching between the immunogenically infected host cells and the host killer T cells. In more detailed analysis of the H-2 region compatibility, it has been shown that the H-2K and H-2D genes, as defined by their "private" serologic specificities, constituted the minimal requirements; thus, H-2K *or* H-2D-matching was sufficient for killing, provided the appropriate non-H-2 antigen was also present (*Blanden* et al., 1975; *Zinkernagel* and *Doherty,* 1975; *Gordon* et al., 1975; *Bevan,* 1975; *Shearer* et al., 1975).

Generally it is held that cytotoxic T cells kill only via H-2K- or H-2D-determined antigens, and if non-H-2 antigens are to serve as appropriate target antigens, then H-2K or H-2D antigens must be shared by the immunogen, the T-killer, and the target as a necessary but insufficient prerequisite for target cell lysis.

This general statement of the phenomenon of H-2 restriction has been rationalized within the framework of the two general models outlined below. By testing the logic and implications of each model against the accumulated experimental data, I hope to show why they are inadequate and thus establish the necessary rules required to construct a new model.

1.2.2. Intimacy Model

Prior to the elucidation of H-2 restriction in T killer effector function, another form of restriction had been extensively documented in terms of physiologic interaction, which is controlled by the Ir-1 gene region and affects T cooperator effector function (*Katz* and *Benacerraf,* 1973; *Katz* and *Benacerraf,* 1976). *Zinkernagel* and *Doherty* (1974b) translated the physiologic interaction theory into the framework of H-2 restriction by considering the H-2 glycoproteins to be self-complementary, for in order for the T cell receptor to contact antigen sufficiently close to deliver the killing signal, the H-2 antigens must complement (see Fig. 2). In this form the intamacy model was ruled out by the elegant F_1 experiment of *Zinkernagel* and *Doherty,* 1974b. Rather than describe the particular experiment in detail at this stage, it is sufficient to note that an F_1 hybrid mouse has in its cell surfaces the H-2 antigens of both parents. Since this included killer T cells, it was possible to give a secondary antigenic stimulation with one or the other of the virally infected parental cells and establish whether the resultant killer T cells were restricted according to the H-2 antigens of the F_1 killer or of the parental immunizing cells. The results clearly indicated that restriction was determined by the H-2 type of the immunizing cells. This point will be demonstrated again when discussing the *Bevan* cross-priming experiments (see Table 1). However, as *Zinkernagel* and *Doherty* (1974b) pointed out, the F_1 experiment does not rule out the possibility of having an anti-H-2 recognition structure that is clonally distributed on the killer T cells; nontheless, it did rule out any model of self H-2 complementarity. Thus, these authors turned to a new interpretation based on the possibility of virally induced changes in the self H-2, or the altered self concept.

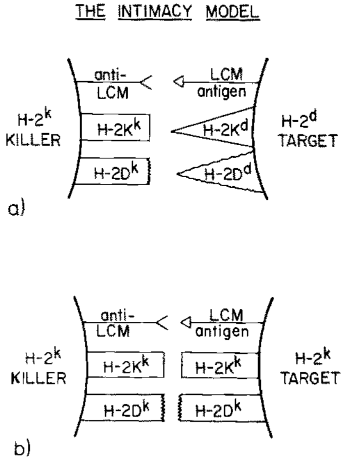


Fig. 2a and b. Intimacy model. (a) H-2^k-H-2^d mismatch: no lysis. (b) H-2^k-H-2^k match: lysis

Table 1. The Bevan cross-priming experiment

Strain immunized	Primary in vivo immunizing cells	Secondary in vitro immunizing cells	Target cell lysis B10	B10.D2	Data line
C.B x C	B10	B10.D2	-	+	2
C.B x C	B10.D2	B10.D2	-	+	3
C.B x C	B10.D2	B10	+	-	4
C.B x C	nil	B10	-	-	5
C.B x C	nil	B10.D2	-	-	6

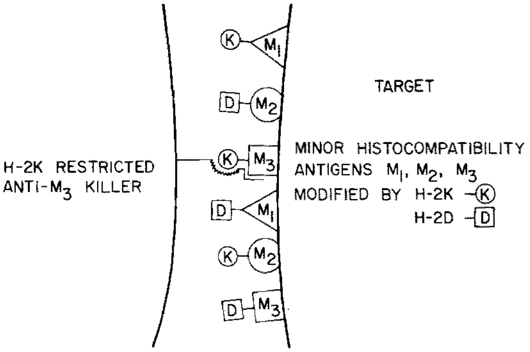
Note: 1. The C.B x C F_1 mice are H-2^b/H-2^d heterozygous on the Balb/c background.
2. B10 is H-2^b and thus H-2 compatible with the F_1 but differs in the background antigen from Balb/c.
3. B10.D2 is H-2^d and otherwise similar to B10.

1.2.3. Altered Self Models

The model was initially proposed by *Zinkernagel* and *Doherty* (1974b) in response to their finding of H-2 restriction in killing virally infected target cells. Two variants were suggested: One was that H-2 became modified by the virus, the other was that a complex association existed between H-2 and a viral antigen, which together altered the appearance of the self H-2 antigens. It is convenient to discuss these variants separately as modified-altered self and complexed-altered self. However, it should be emphasized that the importance of alterations in H-2 is determined primarily by the killer T cell receptor since all other antigenic alterations are presumably ignored. The origin of this restricted recognition spectrum of killer T cell receptors has not been satisfactorily rationalized by any of the theory's adherents.

1.2.3.1. Modified-Altered Self. A *chemical* alteration in the structure of H-2 due to viral infection is certainly feasible; however, as a more general explanation, this model is improbable. *Bevan,* (1975a,b) showed H-2 restriction in cytotoxic T-cell killing directed against many different minor histocompatibility antigens. The assumption that each of the more than 100 minor histocompatibility products alters H-2 in a unique way implies that the H-2K and H-2D antigens are, in effect, encoded by many H-2 and non-H-2 genes; and this is clearly paradoxical. Nevertheless, the converse may still be invoked; H-2 in fact may chemically modify minor and viral cell-surface antigens. In general terms, H-2 modifies non-H-2. Figure 3 illustrates how such a model accounts for H-2-restricted killing. This model requires several ad hoc rules: that the H-2 gene locus codes for a specific modification system as well as for the H-2 glycoproteins, that H-2K does not modify H-2D, that any non-H-2 antigen cannot be modified by both H-2K and H-2D, and that the T cell recognizes only modified cell-surface antigens by including the

Fig. 3. Modified-altered self model



H-2-determined antigen in the recognition complex. The primary experimental evidence against this model is the paradox arising from the F_1 experiment of *Zinkernagel* and *Doherty* (1974b) and the cross-priming effect.

The *Bevan* crosspriming experiment (*Bevan,* 1976b), summarized in Table 1, is based on the use of minor histocompatibility antigens as the immunogen within the framework of the F_1 principle established by *Zinkernagel* and *Doherty.* (This was discussed under the Intimacy Model). The F_1 hybrids (C.B X C) are heterozygous only at H-2 and can be immunized against the B10 minor histocompatibility antigen differences without H-2 incompatibility by cells of either B10 or B10.D2 congenic mice. The first data line of Table 1 shows H-2-restricted killing, similar to the *Zinkernagel* and *Doherty* experiments. Thus, (C.B X C) F_1 mice immune to B10 (H-2^b) antigens kill only the H-2 identical B10 targets and not the nonidentical B10.D2 (H-2^d) targets. The same principle holds in line 4 for B10.D2 antigens and targets. However, the cross-priming effect seen in lines 2 and 3 suggests that the priming antigen need not have the same H-2 as the secondary boosting antigen. Under conditions of the modified-altered self model, B10 minor antigens would be H-2^b modified (as shown in Fig. 3) and thus different from H-2^d modified B10.D2 minor antigens. Therefore, the modified-altered self model would not accommodate the cross-priming data, and H-2-restricted killing. This data leaves open to interpretation, however, the use of a complexed-altered self model.

1.2.3.2. Complexed-Altered Self. In this model non-H-2 antigens (viral or otherwise) become closely associated with the H-2 molecules (see Fig. 4) in order to create new antigenic determinants. This formulation overcomes the clumsy improbability of chemical modification and allows the H-2 and non-H-2 antigens a measure of independence on the cell surface. Using this model to interpret the Bevan cross-priming experiment, it would be possible to have the minor histocompatibility antigens of the B10-immunizing cells removed from their association with H-2^b and presented instead on F_1 host

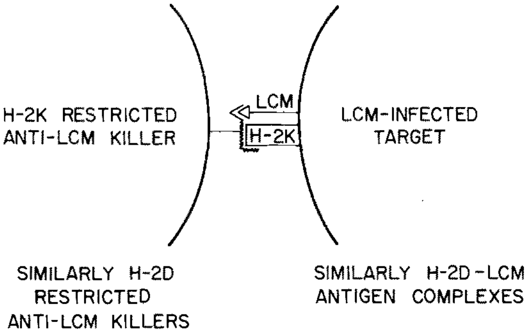


Fig. 4. Complexed-altered self model

macrophages which carry both H-2^d and H-2^b antigens. Thus, priming would occur when minor B10 antigens became reassociated with the H-2^d and H-2^b F_1 host antigens, and so allow F_1 T cells to be primed against B10 antigens in both H-2 complexes. However, there remains the ad hoc requirement at that T cells recognize only the H-2 antigen complexes. At the risk of laboring a point, it is nonetheless worthwhile to examine some of the approaches taken in explaining restriction in T-cell receptor specificity.

1.2.4. Two Functions – One Receptor

It seems increasingly clear that H-2 restriction, even in the broader sense of allo-H-2 reactivity, carries a requirement that T cells recognize something special about H-2-ness; however, recognition of H-2 itself is insufficient to account for specificity in killing non-H-2 target antigens. On the whole it is H-2 restriction of killing these non-H-2 target antigens that has most biologic relevance, and therefore allo-H-2 killing must be given secondary consideration. If the T-cell recognition structure is to be considered a single unit that recognizes a single antigenic moiety, then both the receptor and the antigen must be made up of two parts representing the two functions. The antigen is either a physical or a chemical complex of H-2 plus non-H-2 elements, which is not impossible to rationalize, and is indeed easier in the case of a physical association between the H-2 and non-H-2 components. However, to have a receptor that recognizes neither H-2 nor non-H-2 but only the complex requires a nontrivial rationalization. The negative selection theory of antibody diversity proposed by *Jerne* (1971) might be adapted to provide a potential rationale for H-2 restriction in a single T-cell receptor (*Bevan,* 1976a).

The argument would develop along the following lines. First, a series of inherited (germ-line) genes codes for the T-cell receptor, and the specificity of this repertoire is determined by the species alleles of H-2. Virgin T cells are initially geared to proliferate upon recognition of self antigen (i.e., H-2); then there is a reversal, after which proliferation continues only in the *absence* of recognition. The overall idea is that somatic mutations which diminish the strength of recognition accumulate until only these T cells which no longer recognize self H-2 proliferate and dominate the population. Providing there is only a small reduction in "recognition strength," it is argued that the receptor will retain some recognition potential for self H-2; although alone it is insufficient to be functional. Thus, virtually all T killer receptors will have a residual memory of their anti-self H-2 past and will therefore prefer antigenic structures which contain H-2-ness.

The general principle is not entirely without merit, but in detail the problems multiply to improbability. Put in terms of the usual H-2 terminology, the key problem of detail is in explaining why the "private" specificities of H-2K and H-2D are selected as the "remembered" parts of H-2 during random mutation away from H-2 recognition. In other words, mutation away from H-2 recognition cannot be random; instead mutations must be directed so as to eliminate recognition of public H-2 determinants and still retain a residuum of specificity for private H-2 antigens. Unfortunately, this approach retains too much of the flavor of a germ-line encoded self-nonself discrimination, which is ruled out (see Section 1.3). Perhaps further refinements will improve this negative view, or avoidance, of the selection process. As will be discussed later, there seems to be an easier pathway available via positive selection, i.e., clearly and unequivocally to recognize self H-2, and the private specificities at that.

1.3. Self-Nonself Discrimination Problem

In view of the current popularity of the altered self models regarding H-2 restriction, it is worthwhile briefly to review the self-nonself discriminative process. Implicit in H-2 restriction is an unselected and necessarily intrinsic predilection of the T-cell receptor to confine its attention to H-2 antigenic variants. To argue that the T-cell receptor has restricted specificity for variants of the self H-2 reduces the self-nonself discrimination to a germ-line encoded event. Experimentally, it is unequivocal that the self-nonself discrimination must be learned and cannot be germ-line encoded.

The point is amply illustrated in a familiar example. Consider two parents A and B who can mount an immune response to each other's constituents (e.g., reciprocally reject skin grafts). The offspring F_1 (A × B) will have inherited the potential to react against A and B constituents from the parents, yet the F_1 is in fact made of A and B constituents and does not succumb to autoimmunity. Providing A and B parents are homozygous, the F_1 progeny will not reject parental skin; however the F_2 generation ($F_1 \times F_1$) will almost always reject skin from A or B parental strains, which shows that the potential for reactivity, not expressed in the F_1, reappears in the F_2. Consequently, the self-nonself discrimination is learned, not coded in the germline.

If self H-2 is critical in the determination of specificity for H-2 restricted T-cell killing, the T-cell receptors must be selected on the basis of their ability to recognize self H-2. To have a receptor which alone cannot recognize self H-2 requires the existence of a paradoxical situation in which self H-2 is learned in the absence of recognition; this implies a germ-line determination of self. Although it is possible to construct ad hoc mechanisms that preserve the altered self models and resolve the paradox, there is at least the advantage of clarity and, indeed, a measure of novelty in analyzing the dual recognition model which proposes two physically separate receptor molecules – one for self H-2 and the other for nonself, both acting in concert during target recognition.

2. New Approach to Understanding H-2 Restriction: The Dual Receptor Principle

From the foregoing discussion several areas of difficulty have emerged in attempting to explore the concepts underlying the H-2 restriction phenomenon. The most extensively studied aspect has been the final effector mechanism in which killer T cells cause the lysis of appropriate target cells. I shall first explore the rationale required to construct a minimal model for the killing process. The assumptions made at this effector level will then be put to the test of biologic rationality in the next section of this essay by asking if H-2 restriction can be considered of evolutionary significance in generating a new concept of the immune system. These considerations will introduce a perspective which, while uncommon today, was the traditional view of an immune system that functioned as a survival kit in combating infectious diseases.

2.1. Rationale for a H-2 Restricted T Killer Cell Effector Mechanism

The duality of specificity in H-2 restriction leads to the need for two recognition structures on the T cell – one anti-H-2 self, the other anti-nonself. Let the anti-H-2 self be symbolized as anti-H, and the anti-nonself as anti-X. The anti-X receptor is selected by whatever mechanism determines the nonself discrimination, i.e., the avoidance of autoimmunity. The anti-H receptor is also selected but on the basis of self-H versus self-non-H, and the selection must, for example, take into account the distinction between H-2K and H-2D. This brings us to the point that anti-X and anti-H must be clonally distributed. For the moment, I will assume these requirements to be fulfilled, though later this will be analyzed in detail.

Consider now the minimal rules necessary to account for the phenomena of H-2 restriction at the effector level. If there is to be more than one receptor of each type on the T-cell surface, the rule must be made that anti-X and anti-H function only as a paired unit; otherwise, there would be no restriction. Since H and X antigens must be present on the same cell (*Gardner* et al., 1974), the coupling between anti-H and anti-X requires a physical pairing in the T-cell membrane.

Delivery of a killing signal cannot be dependent upon a particular signal from the anti-H receptor recognizing H, because allo-H killing takes place without specific anti-H recognition, i.e., allo-killing is not restricted. Thus, anti-X must be the source of the inductive signal for killing. For killing to be successful when only anti-X recognizes nonself-H (H′), a new constraint must be invoked which requires H structures – independent of antigenic specificity – to be distinguished from non-H structures on the target cell. Since anti-X has a specificity repertoire which includes recognition of H and non-H antigens, the distinction between H and non-H structures must be a function of the target per se. The general argument developed here on the basis of separate anti-H and anti-X receptors holds equally well in the case of a single receptor as envisaged in the altered self models. The final structure recognized by the T-cell receptor (s) is determined indirectly or directly by the separate contributions of the independently derived H and X antigens.

A rule is required which states, in principle, that the killing signal can only be delivered at or near the H structure on the target cell. This rule is of paramount importance for understanding the whole phenomenon of H-2 restriction, and without it there is no unified role of H in the killer function of T cells. In the same way that H-2 restriction could not be the sole property of the target antigens, as discussed in the section on the altered self theories, so restriction cannot be the sole property of the killer T cell. To formalize this rule for the purposes of discussion, the killing process can be envisaged as occurring when there is proper alignment of a killer-donor site on the killer T cell (activated by a signal from anti-X) and with a killer-acceptor site (linked to H) on the target cell. The resultant complex is a killing channel. It seems unlikely that the killer-acceptor site on target cells is the H-2 molecule per se since it has been shown that a teratoma cell line (F9) that does not have H-2 and is not normally susceptible to lysis (*Forman,* 1975) can nonetheless be lysed by killer T cells if Concanavalin A is used to "glue" the killer and target together (*Goldstein* et al., 1976).

The overall arrangement of antigens, receptors, and killing channel as described above is shown in schematic form in Figure 5 (a and b); also demonstrated are both successful (c and d) and unsuccessful (e and f) arrangements of structures on T killer and target cells.

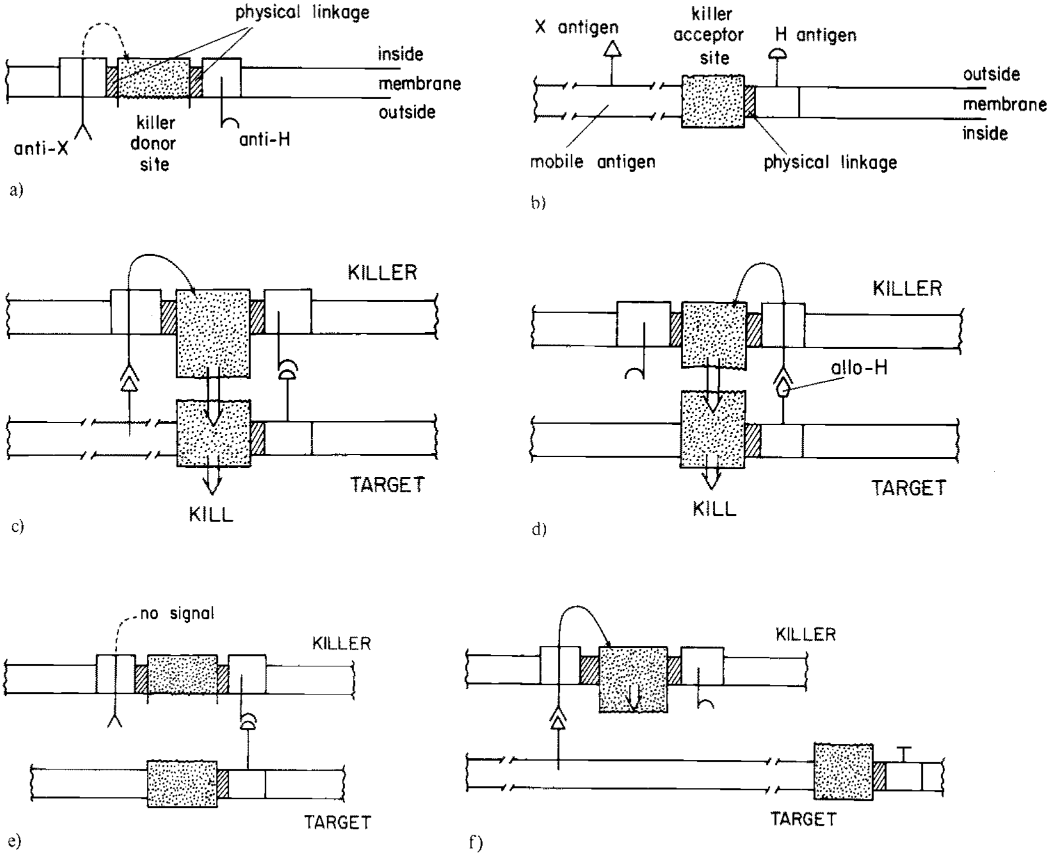


Fig. 5a-f. Dual recognition model. (a) Arrangement of structures on the membrane of killer-T cells. (b) Arrangement of structures on the surface of target cells. (c) Successful: H restricted killing anti-X. (d) Successful: allo-H killing. (e) Unsuccessful: no X antigen – no signal. (f) Unsuccessful: no H antigen – no acceptor

If a common mechanism is considered to operate in both allo-H and H-restricted anti-X killing, the above model must be regarded as minimal; removal of any one component removes an essential element from the complex.

From a practical point of view, some consideration must be given to the question of receptor affinities. Since there are presumably many copies of the T-cell receptor and as many copies of H and X antigens on the target, a multipoint binding interaction can occur which will be cooperative and of high binding affinity. In general, it must be argued that anti-H and anti-X receptors, separately or together, do not form tight binding complexes. This constraint is necessary in order to accommodate the "cold-target" inhibition studies which form the most frequent argument made against the dual-recognition or two-receptor model. In brief, it is the failure of X antigens on H-2 incompatible targets to compete with the same X antigens on H-2 compatible targets in the effector phase of the killer T-cell response. Experimentally, it has been shown that, for example, LCM-immune Balb/c (H-2^d) spleen cells, which lyse LCM-infected H-2^d target cells as measured by the release of intracellular ^{51}Cr isotope, are equally inhibited in their activity by unlabeled LCM-infected CBA (H-2^k) cells and unlabeled uninfected Balb/c cells (*Zinkernagel* and *Doherty,* 1975). However, it is clear that some weak inhibition occurs with both kinds of inappropriate target, and so far as the two-receptor model is concerned, both receptors can be regarded as equivalent; thus equal inhibition by separate H-2 and X antigenic competitors is to be expected. The argument therefore becomes unconvincing, and the inhibitory effects reflect a biologic necessity that anti-X have low affinity for free X antigen and that anti-H does not leave the T cell permanently attached to the target and thus unable to kill repeatedly. In all likelihood the formation of a killing channel will be a dominant contribution to high-affinity binding; some strong interaction is required to cause binding when anti-H or anti-X do not perform this function.

The dual receptor model of T killer effector function outlined here invokes a number of constraining rules, and certainly in the absence of the discussion to follow, they represent a weighty collection of assumptions that were not surprisingly considered excessively ad hoc by *Zinkernagel* and *Doherty.* However, it is worthwhile to point out that the present model and accompanying rationale provide the first comprehensive analysis of a T killer mechanism, and many of its aspects are amenable to experimental test. Listed below are the key rules invoked to make the dual receptor model workable; no importance is attached to the order because the rules are interlocking.

1. There are three elements in the receptor complex on T cells – anti-H, anti-X, and the killer-donor site.
2. The receptor complex is functional only when all three elements are in close physical proximity.
3. The killer-donor site of the T-cell receptor complex is inaccessible until activated by a signal generated by anti-X that binds an appropriate X antigen.
4. The H antigen on target cells is either itself the killer-acceptor site necessary for effective delivery of a killing signal to the target cell, or is closely linked to the site.
5. The affinity of anti-X and anti-H, either separately or together, for target cell antigens is low relative to the binding mediated by formation of an effective killing channel via killer-acceptor and donor site interactions.
6. The anti-H receptor has the specificity necessary to distinguish murine H-2K and H-2D antigens in the one haplotype pair and the alleles of H-2K and H-2D in H-2 heterozygotes.

7. On any particular killer T cell the anti-H receptor has a unique specificity that recognizes either H-2K *or* H-2D but not both, i.e., the anti-H receptor is clonally distributed.
8. The anti-H receptors have a specificity that has learned to be antiself H, irrespective of whether self is defined genetically (from conception) or functionally (in the case of acquired tolerance).

2.2. Two Functions – Two Receptors

The use of separate receptors for separate functions, as outlined here under the dual recognition principle, allows greater scope and flexibility in approaching the problem of selection, which determines that anti-H is antiself H. A means of selecting antiself H will be discussed in detail Section 3.3.6, along with the question of distinction between H-2K and H-2D. Minimally, it would seem that one H antigen should be sufficient, and yet there are two gene loci; a sharp distinction is made in keeping H-2K recognizably different from H-2D. Clearly, there must be some reason for having two loci with similar structures and functions but antigenically different. Since the H-2 restriction phenomenon has shown for the first time a biologic use of H-2K and H-2D differences, it seems worthwhile to see if this can be incorporated into a conceptual framework. The development of such a general framework has been guided by the belief that an immune system – such as that observed today in vertebrates – arose from a more primitive ancestor during a process of mutation and selection. Thus, we turn to the possible evolutionary origins of a cell-mediated immune system.

3. Evolution of a Cell-Mediated Immune System

3.1. Background

The division of transplantation histoincompatibility antigens into major and minor classes has both practical and theoretic importance. This section of the essay will explore the evolutionary process which may have resulted in these major and minor categories in terms of more general immunologic phenomena. Although there is an obvious complexity of genetic organization and function within the major histocompatibility locus, it is reasonable to assume that this complexity arose from a simpler structure in order to meet some need for survival. The most convenient and simplest starting point is the phagocytic feeding process found in unicellular eukaryotes, such as amoebae. A frequent threat to their survival is likely to be infectious agents, such as bacteria or viruses. From this starting point, it is possible to envisage the genesis of an immune system during the evolution of vertebrates. It is beyond the scope of this essay to discuss the evolution of an entire immune system; however, the phylogenetically more primitive component which falls under the heading of cellular immunity can be profitably examined. Some prefatory comments may prove helpful in providing an orientation, considering my unusual approach, which places emphasis on an evolutionary process driven by selection due to infectious disease.

3.2. Division of the Immune System: Cellular Versus Humoral

It is to a purely physiologic approach that we owe the classification of lymphocytes on the basis of their thymic and bone marrow origins, abbreviated T and B cells. A more practical division of the immune effector function has been made from experiments on the passive transfer of immunity, which showed that immunity could be conferred by serum (humoral) in some cases, while in others it was transferred only by lymphoid cells (cellular). The motive for examining the methods of passively transferring immunity came from a clinically oriented concern for fighting infectious diseases. As a rule and to illustrate the approach used here, infectious disease can be categorized as due either to intracellular or to extracellular parasites. The passive transfer of immune serum is effective in treating extracellular parasitism, whereas immune cells are required for intracellular infection. The model systems developed in mice which demonstrate these principles are (1) pneumococcal infection of the extracellular alveolar spaces in the lung that requires for resistance antibodies to the pneumococcal polysaccharide (*Wood,* 1953), and (2) *Listeria monocytogenes* infection of macrophages which requires immune T cells for resistance (*Blanden* and *Langman,* 1972; *Blanden,* 1974). These two examples have been chosen because it is clear that humoral factors, i.e., antibodies, are totally without effect on the course of infection with *Listeria;* conversely no cytotoxic T-cell elements have proven effective in modifying the progression of a pneumococcal infection. It is also interesting to note that both pneumococci and *Listeria* have mechanisms of resistance to phagocytic destruction, and it is only through immune intervention that these parasites are rendered destructible. With the exception of complement-mediated cytolysis, which is of questionable efficacy in resisting any common infectious agent, the phagocytic process remains the primary means of destroying potentially lethal infectious agents.

I have taken as fundamental the assumption that resistance to intracellular parasites requires cellular or T-cell immunity and that resistance to extracellular parasites requires humoral immunity. The thrust of my argument will be that intracellular parasites were the selective agents which led to the evolution of a cellular system. Any selection caused by neoplastic disease in shaping the immune system I have regarded as zero; this is not a simplifying assumption, and in addition there is at present no compelling evidence to connect the control of neoplasia with immunity. Although I will not discuss humoral immunity in this essay, it is a logical extension of the analysis.

3.3. Evolutionary Process

3.3.1. Macrophage

Evolutionary processes in multicellular organisms require that selection pressures be expressed in the survival of organisms as individuals or single genetic units. Several attempts (*Theodor,* 1970; *Bodmer,* 1972; *Burnet,* 1973; *Hildemann,* 1974) have been made to trace the origins of the self-nonself discrimination by the immune system in terms of the need to keep units of genetic identity together and separate from nonidentical neighbors. However, the problem of self-nonself separation at this level is superficial and bears little resemblance to the systemic problem that infectious diseases pose. A more likely starting point comes from the feeding process, where nonself particles are encapsulated in digestive vesicles, a process well suited to removing and destroying infectious particles. The idea of phagocytosis being important in immunity has a long history, but macrophages do not show the precise specificity distinctions which characterize the immune system. However, an ideally suited candidate from which to select for the present day immune system is the macrophage. The self-nonself discrimination made by macrophages early in the evolution of multicellular organisms will now be considered. Although the term macrophage has a rather precise meaning in describing a particular class of phagocytic cells, I have taken a much broader meaning throughout the ensuing discussion; here I consider the macrophage as a prototype for all classes of cells with phagocytic properties.

3.3.2. H and Anti-H System

The assumption is that primordial macrophages had a self-nonself discrimination mechanism, which in its simplest form required that self be specifically and particularly recognized. Self-recognition by the macrophage must have signaled nonphagocytosis, allowing all other substances to nonspecifically induce phagocytosis. The self-marker is designated as H and its recognition structure on macrophages, as anti-H; all members of a species would have the same H and anti-H. Under these conditions, macrophages could ingest and digest all particulate parasites not carrying the self-H marker. But if a parasite were to infect a cell and grow intracellularly, it would be under the protection of the H marker on the host cell surface and thereby avoid phagocytosis.

3.3.3. Defense Against Intracellular Parasites Demands Anti-X

Unless an intracellular parasite were to alter the cellular H marker, it would be protected from phagocytic attack while in the intracellular state. Consequently, intracellular parasites would tend to avoid modification of the H marker. Animals with macrophages that had acquired the capacity to recognize infected cells would be at a strong selective advantage.

Such considerations as these suggest that macrophages evolved a mechanism for specific recognition of nonself, under selection imposed by intracellular parasites; this nonself recognition structure is called anti-X. It seems likely that anti-X arose as a duplication of the genes, coding for anti-H followed by mutation to anti-non H (or anti-X). A closer analysis of this sequence introduces some difficulty, because an interaction of H with anti-H signals "off" for phagocytosis. If anti-X simply replaced anti-H, this would be of negative selective advantage. However, an enormous selective advantage would follow if anti-X was coupled to the "on" signaling system. Once an "on" signaling anti-X evolved, there would follow a rapid expansion of the anti-X repertoire to track various escape patterns presented by intracellular parasites.

At this point it is useful to look more closely at the response of macrophages to the signals they receive from interactions between their receptors – anti-H and anti-X – and the infected cells which carry H and X antigens. It is unlikely that an anti-H interaction signaling "off" controls the phagocytic behavior of the entire macrophage surface, because a macrophage which sits on a substratum of self would be paralyzed over all of its surface and could not function. Therefore, "off" signals, generated via anti-H, are expected to have only a local effect in overriding the nonspecific "on" signal. Because the X and H antigens on infected cells are expected to be in close proximity, anti-X must be connected to the "on" signaling system at a level beyond that at which an anti-H interaction could create an effective "off" state. Perhaps the best way to imagine this signaling system is illustrated in Figure 6, in which phagocytosis is signaled by surface contact, through an intermediate stage to the actual "on" generator. The anti-H would cause a block in signal transmission between the nonspecific sensor and the "on" generator. Then, if anti-X were connected directly to the "on" generator, its signal would bypass any block set up by anti-H. A hierarchy of signals is postulated with nonspecific "on" blocked by anti-H and specific "on" dominant over anti-H "off".

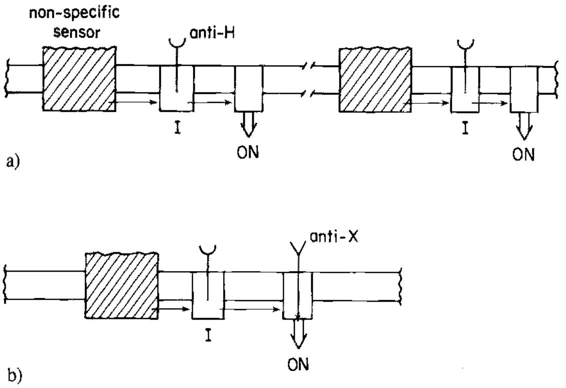


Fig. 6a and b. Signaling phagocytosis in macrophages. (a) The primitive anti-H and 'off'. (b) Anti-X and the specific 'on'

This primitive immune system would select against intracellular parasites, unless they produced X antigens that fell into the self category, i.e., parasites would tempt the immune system to self destruction. Therefore, the discrimination of self from nonself must be learned and the anti-self specificities eliminated from the anti-X repertoire, without eliminating the individual.

3.3.4. Anti-X as Anti-Self Demands Clonality

Clonality means that one cell expresses only one specificity of receptor; this was first assumed in the clonal selection theory (*Burnet*, 1959). At this stage, we encounter clonality as a necessity in evolution and as a solution to the problem of somatically eliminating one specificity – that of anti-self – from a large repertoire.

The introduction of clonality among the anti-X receptors as a prerequisite to exercising the self-nonself discrimination decision clearly dissociates the anti-X receptor from the macrophage discussed above. The new cell type with clonally distributed receptors will be called in anticipation cells of the thymus-derived (T) lineage. In the following Section 5 dealing with the self-nonself discrimination mechanism, we will have occasion to note a special role for the thymus as a discrete organ found in all vertebrate immune systems studied so far; hence, the symbol T will stand for thymus-derived cells.

Although the central issue at this stage is a solution to the problem of anti-X being potentially anti-self, we must consider what the role of anti-H might be during this reassortment of anti-X receptors into a clonal array. On the one hand, anti-H as the preserver of self integrity becomes redundant if the specificity of anti-X must be selected on the basis of self versus nonself reactivity, and it might be argued that anti-H is therefore no longer required. However, on the other hand, anti-H can provide useful information as a part of the recognition system of T cells in helping to distinguish cell-bound from free forms of the X antigen. In this latter context, faculative intracellular parasites might predominate in the extracellular form and thus divert the attention of T cells from their primary target, namely infected self cells. Furthermore, an a posteriori argument is that H-2 restriction has been established as an essential property of today's vertebrate immune system.

Having dissociated specific recognition and phagocytic destruction, we can speculate on how these mechanisms might be reconnected. For example, consequent to encountering antigen, there is a class of chemotactic factors elaborated by T cells that would be excellent candidates for connecting T cells to macrophages. At present there is little information on the possible evolutionary origins of the lytic activity of effector T cells. Although T-cell lytic activity is considered by some to be an in vitro artifact, there seems good reason for such an activity in vivo, namely as a means of releasing parasites from infected cells into a macrophage-rich environment.

Any mechansim of self-nonself discrimination requires two pathways of expression for a given set of specificities – one pathway must lead to unresponsiveness for self tolerance and the other, to responsiveness for antiparasitic immunity. Given our present knowledge of the unidirectional flow of structural information from genes to products, it is impossible to arrange on the cell surface a multiplicity of receptors which differ only in specificity and then have one or more lost specifically while retaining the others. In order to allow selection of a particular pathway of response for each receptor, it is necessary to have one receptor per cell; thus, the cell as a whole is signaled via its receptor to either responsiveness or unresponsiveness as dictated by the mechanism of self-nonself discrimination.

3.3.5. Mechanism of Self-Nonself Discrimination

It is assumed that the self-nonself discrimination cannot be encoded in germ-line genes but must be learned. Additionally, self antigens cannot be intrinsically nonimmunogenic nor different in any other physical or chemical property from nonself antigens, because what is self for one individual is nonself for another. The only distinguishing property of self antigens is their temporal relation to the immune system. Self exists prior to the appearance of immune recognition during ontogeny and persists throughout the life of the individual. In contrast, nonself antigens enter the individual subsequent to the appearance of the immune system and are transient. It is the prior and persitent property of self – in contradistinction to the subsequent and transient property of nonself – that provides a basis for the learning mechanism.

Having established the principle of a self-nonself discrimination, the question to which we now address ourselves is the detail of the mechanism which allows cells to be rendered responsive or unresponsive toward X antigens, depending on whether X is nonself or self. The discussion is best pursued in two parts as it pertains to signaling and to the thymus.

3.3.5.1. Signaling System. The states of unresponsiveness and responsiveness are considered to be the final outcomes of a discriminative process; however, the initial state in which cells are "born" must be one of responsiveness. Although it has been argued on several occasions (*Lederberg,* 1959; *Bretscher* and *Cohn,* 1968; *Bretscher,* 1972; *Coutinho* and *Möller,* 1975) that the minimal pathway of receptor signaling should be for unresponsiveness, it seems difficult to envisage the selection of this minimal "off" signal without first having an "on" signal. In an earlier part of this essay (Sect. 1.3), it was argued that the self-nonself discrimination must be learned, and it was apparent that self reactivity was suppressed or deleted, leaving all other nonself reactive cells in their native state of reactivity. Thus, I assume that killer T cells evolved by, first, having clonally distributed the anti-X receptor and, secondly, having substituted killing in place of the phagocytic process.

In the simplest terms, unresponsiveness is cell death, and the killer effector function constitutes a means of delivering a signal for unresponsiveness. The question is now reduced to whether self-reactive T cells can be killed, and nonself-reactive T cells can be allowed to survive.

Consideration can now be given to the differences between major and minor H antigens in the self series. The dedinition of H initially dependent on two criteria: (1) its presence on all cells, and (2) most importantly, the presence of anti-H to protect the self from phagocytic destruction. The introduction of a T cell made it necessary to evolve a cytolytic effector function in order to connect recognition back to intracellular parasitic destruction. The cytolytic effector function is intimately tied to the H molecule, and as argued earlier, the killing signal can only be delivered at a site determined by the presence of the H molecule on the cell surface.

Although the idea of elimination of self-reactive clones in the thymus is by no means novel, having been suggested by *Burnet* in the context of his clonal selection theory, the mechanism arrived at here, by considering a possible evolutionary pathway, can provide a clear rationale for these previous assumptions.

3.3.5.2. Special Role for the Thymus. If newly arising T cells first acquired receptors and reactivity within a confined organ, then any self-reactive T cell would be limited in the range of its destructive potential. The thymus is known to be essential in the genesis of cell-mediated immune effector cells and is an organ common to all vertebrates with immunologic potential. Thus, it is to be anticipated that the thymus arose in order to function as a cell-tight compartment in which T cells could acquire activity and be selected on the basis of self and nonself reactivity. Should a newly arising T cell have anti-self reactivity, it would be expected both to kill surrounding cells and to proliferate. The expression of this destructive potential is at first glance deleterious, for when such a self-killer divides to give two daughters, the obvious proximity of these cells after division makes mutual destruction a high-probability event. There would be thus a simple and effective mechanism of self-nonself discrimination achieved by the stratagem of containing emergent T cells within an enclosed compartment. In principle, self-reactive T cells would be eliminated by turning the lethal effector function back upon itself. It could be postulated that, as a rule, T cells must spend a period of quiescence free from proliferation stimuli before they can emigrate to the extrathymic periphery.

The condition that *self* is present *prior* to the genesis of reactive T cells and is also *persistent* applies to antigens present in the thymus, but more importantly, these antigens must be present on effector T killer cells. *Subsequent* and *transient nonself* antigens will not be incorporated readily into the thymus, nor will such antigens persist there; however, nonself antigens will be present in the periphery, and when cell bound, they will attract the attention of killer T cells. So long as antigen in the extrathymic areas is not bound to killer T cells, the pattern of elimination found in the thymus will not occur, and immunity will develop as required. However, should antigen be bound to a mature T cell in the extrathymic periphery, it is not anticipated that these cells will be induced to proliferate in the absence of anti-H-dependent recognition. The peripheral T cells which bind free antigen may be eliminated if other T cells can recognize the cell-bound antigen. It is conceivable that such a situation would give rise to the phenomenon of suppressor T cells, and indeed there is some evidence (*Epstein* and *Cohn,* 1976) that certain types of suppressor T cells are H-2 restricted in their activity.

Thus far, the argument has remained focused on the need for a clonal distribution of the anti-X receptor and for the elimination of anti-X specificities which are anti-self. With respect to the anti-H component, it was previously stressed that H-2 restriction required the selection of anti-H self; this will now be discussed in terms of our evolutionary framework.

3.3.6. Selection of Anti-H as Anti-Self H

The challenge to devise a selection mechanism that allows self-H to be singled out from the array of all self antigens is differnet from that posed by the self-nonself distinction. However, given a receptor capable of specific recognition, selective methods can be envisaged. It was previously pointed out (Section 1.2.4) that models of restriction requiring a single T-cell receptor that recognizes neither H nor X but the interaction between them leads to a situation in which selection of self H is not possible because recognition as such is excluded. Under the dual receptor model, the question that must now be considered is how the selection process might operate to give an anti-H receptor directed against self-H.

It is possible to extend the principle used in analyzing the self-nonself discrimination, namely the property that two components can be separated both in time (prior self versus subsequent nonself) and in location (persistent-thymic self versus transient-peripheral nonself). The following sequence could be reasonably envisaged as a selection mechanism that distinguished H from non-H within the constellation of self antigens. Minimally, two compartments are required through which nascent T cells migrate on the basis of continued recognition via a receptor operating in the anti-H position. The compartments would be made of self components, and if the only antigen common to the two compartments was H, the only T cells capable of traversing the two compartments would be those with anti-self-H receptors. The use of only two compartments is perhaps too simplistic, and in fact several may be required to obtain optimal conditions. The overall effect would be analogous to affinity chromatography, where selection would favor T cells with anti-H receptors that have anti-self H specificity; the affinity of receptors for H should not be so high as to remain attached to the compartmental antigens. Experiments with radiation chimeras and adult tolerance in the analysis of H-2 restriction clearly show that tolerance leads to a state of accepting the nonself as if it were self, and H-2 restriction in tolerized or chimeric animals expands to cover the new H-2 antigens (*Zinkernagel,* 1976; *von Boehmer* and *Haas,* 1976; *Pfizenmaier* et al., 1976). If further proof were needed on the learned nature of anti-self H specificity, these tolerance experiments must be considered excellent confirmation.

Thus far, the genetics of the H-2 complex and its ancestry have been only briefly alluded to in order to stress the selection pressures necessitated by the challenge of intracellular parasites. A more detailed analysis of the evolutionary pathway is required at the chromosomal and genetic levels.

3.3.7. Evolution at the Gene Level

In a species having only one H and one anti-H, the self-nonself discrimination is not necessarily learned; it can be germ-line gene coded. This carries two important constraints: (1) a mutation in either H or anti-H leading to a failure in recognition must be lethal, and (2) all members of an interbreeding unit or species must have the same H and anti-H specificities. The H and anti-H genes need not be linked. Considering the critical importance of a functioning H and anti-H system in controling phagocytosis, it is possible that multiple sets of H and anti-H existed as backup or fail-safe arrangements. The phenomenon of antigenic modulation or phase variation in *Paramecium* might represent a series of H antigens with correspondingly predicted anti-H receptors. In the context of genetic redundancy, this possibility provides a multiplicity of genetic sites which can mutate and be tested for quantitative efficiency in survival value without placing the organism as a whole at the qualitative extremes of life or death.

The remainder of this section on genetics will be grouped under two headings: (1) anti-X and the "on" signal and (2) the concept of variable and constant genes with a somatic generation of diversity – the "little bang."

3.3.7.1. The Transition From Anti-H to Anti-X and the "On" Signal. Starting with anti-H as the minimal assumption, the steps of gene duplication and mutation can be considered as intrinsic properties of DNA (*Smith,* 1976). However, whenever a variant of anti-H arose which could combine with a pathogen, the consequent "off" signal that developed could be lethal if the macrophage failed to ingest and destroy the pathogen. At this point, it is essential to appreciate the multifocal nature of evolutionary change. The generation of multiple copies of anti-H and mutated variants must be considered as constantly occurring, and the variants per se are unselected. Particular variants of anti-H, hereafter called anti-X for clarity, will be subject to selection if their specificity coincides with an antigen carried by a pathogen. As long as the anti-X receptor specificity is associated with the anti-H type "off" signal, the pathogen carrying an X antigen will be at a selective advantage, and the host "immune" system is at a distinct disadvantage. There are two mutational routes available to the immune system if it is to avoid being placed at a selective disadvantage: One is obvious – a reverse mutation to eliminate the variants; the other is to connect anti-X to the nonspecific "on" signal and thereby give a specific anti-X "on" signal. A detailed analysis of the steps from anti-X "off" to anti-X "on" shows the need for a mutation in the acceptor end of the receptor, i.e., where it is inserted into the macrophage membrane. This change in the membrane acceptor portion of the anti-X molecule is required, because the signals generated via the anti-X and anti-H receptors must be distinguished. In summary, three mutational steps are envisaged: (1) duplication of anti-H, (2) a change in specificity to give anti-X, and (3) a change in the acceptor portion of anti-X that allows anti-X but not anti-H to be connected to the "on" signal for phagocytosis. These three mutational steps must be sequential, and the first two are either unselected, or in the case of step (2) a possible selective disadvantage. Only at step (3) is there an advantage when the anti-X can recognize and promote destruction of the X-bearing parasitized cell. As discussed earlier (see Figure 6), the anti-X will have been inserted into the "on" signal at a level beyond where anti-H blocked the nonspecific "on"-signal transmission. Thus, no further mutational steps are required to have an effective trio of signals.

The creation of anti-X "on" is well suited to recognition and attack on intracellular parasites, and it is critical to the evolutionary pathway considered here. Once established, the anti-X can undergo duplication and mutation to create a repertoire of anti-X specificities. At first the repertoire must be considered as a series of germ-line genes encoding the entire anti-X molecule, thus in any individual representing a genetic record of ancestral encounters with pathogens. A pathogen can mutate the same way the anti-X genes mutated, and as a pathogen changes its antigenic structure from X to X′ by a random mutation, the *anti-X cannot predict the new specificity X′*. Since small intracellular parasites multiply rapidly, the change from X to X′ within the viral population must be considered fast, relative to adaptation by the multicellular host. Although an anti-X of broad specificity would slow the effective rate of viral escape, there will be escapees nonetheless. The point to be emphasized is that multicellular organisms must carry *adaptive* stratagems in the germ-line, while the short-lived, rapidly replicating viral genome relies on wholesale life and death selection following mutation as the adaptive mechanism. Thus, the multicellular individuals with their anti-X germ-line immune system carry the history of viral, or X, experiences, leaving increasingly fewer alternatives in the array of new X antigens that can escape the ponderous weight of anti-X accumulations. Unwittingly, the stratagem of germ-line memory is leading the animal toward self-destruction, as the viral X is being selected to mimic the self antigens. Put another way, anti-X accumulations drive the viral Xs to mimic host antigens, at which point autoimmunity destroys the organism without viral intervention. This is an evolutionary dead-end for both virus and its host, unless the host can adapt in a new way. The virus cannot mutate to preserve the organism, its host, from elimination, because death by autoimmunity is now independent of the virus. Thus, arises the question of self-nonself discrimination. In earlier Sections 1.3 and 3.3.5 the mechanism of self-nonself discrimination was discussed in detail, and in the next section genetic requirements will be dealt with.

The thymus and clonal distribution of receptors are clearly examples of mutation and selection at work, but these changes per se do not come about from changes in either H, anti-H, or anti-X. However, it is clear that clonality does demand corresponding reorganization of the H genetic complex.

3.3.7.2. **Variable and Constant Region Genes**

3.3.7.2.1. The Anti-X Series. The accumulated germ-line memory of anti-X generates a series of highly reiterated genes. As pointed out previously (*Hood* and *Prahl,* 1971; *Smith,* 1976), this situation would favor homologous and unequal crossing over, which leads to gene expansion as well as gene contraction. It seems likely that the range of anti-X specificities will therefore be limited by the need to balance gene expansion and contraction, leaving the germ-line memory with potential gaps. However, the degree of precision in specificity required for an anti-X to successfully recognize antigen need not be particularly great, and gaps in the anti-X repertoire of specificity would be largely covered by a broad specificity of recognition. Limitations in the size of the germ-line gene pool would be balanced by a broad specificity in recognition by anti-X. The confrontation of anti-X recognizing self arises relatively soon in the gene expansion process, if a broad specificity must be maintained to balance the parasite load. A mechanistic solution to the self-nonself discrimination process would have evolved prior to anti-X equaling anti-self H since there are many minor cell-surface antigens and only one or a few H antigens.

The primary problem with a relatively small repertoire of anti-X genes is not the occurrence of anti-X as anti-self specificity but the vulnerability of the constant region of the receptor which sits in the membrane and transmits the signals. A mutation in the constant region of anti-X can at once eliminate the functional use of the immune defense against the multiplicity of parasites, all covered by the one broad specificity anti-X. Selection pressure on point mutations in the antigen-binding region of anti-X will be small in comparison to mutations occurring in the membrane insertion site. Under selection pressure as well as other pressures, it seems likely that the anti-X will split into two genes, one for the constant region, another for the variable, antigen-binding region. With this arrangement, the constant region gene can be conservatively duplicated to provide a backup of redundancy as a fail-safe against mutation.

Following this line of reasoning, it seems likely that the variable and constant region gene system, now established for the immunoglobulins in humoral immunity, has its evolutionary origin in the T-cell receptor molecules. Further consideration of the anti-H gene suggests that inevitable gene expansion and mutation will give rise to multiple anti-H gene specificities.

3.3.7.2.2. The Anti-H Series. The primary role of anti-H was initially to recognize the self H and inhibit the nonspecific "on" phagocytic signal; however, with recognition and phagocytic functions separated by the introduction of T cells, it becomes necessary to reevaluate the role of anti-H (see Section 3.3.4). The anti-H function in T cells was of selective advantage for two reasons: firstly, in helping distinguish infected cells from free infectious agent, and secondly, as a vital component in focusing the killing signal to the sensitive site near H. Changing the functional role of H and anti-H interactions allows corresponding modification of the mechanism of interaction. Regarding the identification of infected cells, the H antigen can be any cell surface component so long as there is a corresponding receptor capable of recognizing it in the anti-H position in the T-cell receptor complex. The role of H in identifying the sensitive site at which the killing signal can be delivered is not a priori necessary in a minimal model of the T-cell immune system and has been brought into the discussion only to accommodate the killer effector function as found in immune effector armory known today. This distinction is made to raise the possibility of degeneracy in the anti-H receptor and leads to a consideration that variable and constant region genes may have been advantageous for anti-H, paralleling the argument for anti-X.

If variable and constant region genes evolved for anti-H and anti-X, it would become feasible to envisage a merger of variable region genes into a single pool and to retain separate constant region genes for anti-H and anti-X that maintain the separation of their functions. The situation is illustrated in Figure 7. Clearly, the arguments are tenuous at this stage, but they lead to a situation which allows adequate rationalization of several otherwise puzzling features of T killer activity, which are currently documented though poorly understood.

One of the major limitations in effectiveness of the primitive T killer system was its reliance upon germ-line memory of pathogenic X antigens, which carried the compromise of limited precision in specificity of recognition. In principle the system could be said to lack flexibility, adaptability, or as *Cohn* (1970) has called it, anticipatory potential. The arguments

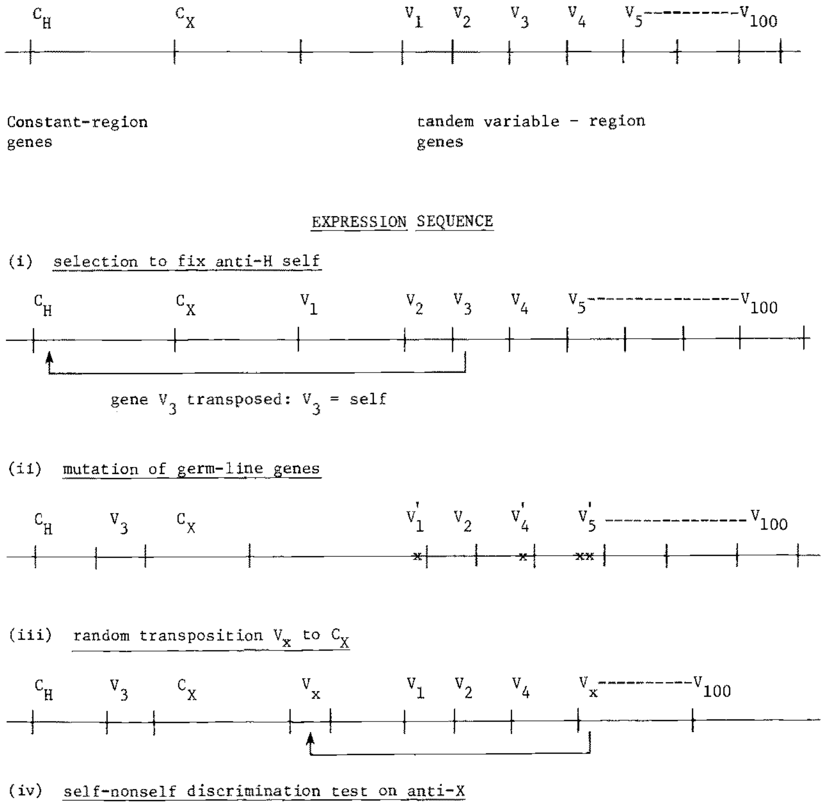


Fig. 7. Genetic organization of anti-H and anti-X

have been well documented for the immunoglobulin variable region genes, having a degree of variation generated somatically from a limited repertoire of germ-line genes. Parallel arguments can be made for the T-cell receptor molecules and their variable region genes. However, the concept can be expanded a little further in the case of anti-X and anti-H germ-line variable region genes to include the selection pressure which maintains the germ-line gene structure and function.

3.3.7.2.3. Germ-Line V Genes Maintained as Anti-H (Species). The process by which anti-H is selected as being anti-self H is in essence a case of trial and error. In contrast to the anti-X specificity which is different in every T cell, the anti-H is essentially constant (at most, there are four anti-H specificities in F_1 mice). The repertoire of variable (V) gene specificities will determine the probability of a randomly selected V gene being anti-self H. If the probability of selecting an anti-self H is to be better than "one in a million," the germ-line V genes should be maintained to recognize the species H antigen alleles. Somatic variation should be delayed until after the anti-H specificity has been selected and should operate on the unused germ-line V genes. To impose the condition that mulation be delayed until after anti-H selection implies that mutation cannot be random and spontaneous. The jargon used in discussing the immunoglobulins has been to contrast mutation and selection versus the big bang; here I would prefer to have the controlled mutation mechanisms termed "little bang," to imply only a limited amount of variation.

The little bang, generating limited variation, is proposed in order to allow a reasonably high probability (say 0.5) that any particular V gene will not be mutated. Under conditions such as these there would be a high frequency of T cells expressing an anti-X that was a germ-line V gene specificity, and these would be anti-nonself H. In this way a rational argument can be made to explain the high frequency of allo H reactive T cells. As many as 50% of mouse spleen cells present after treatment with Con A may be allo H-2 reactive T killers, and about 3% appear to be specific for a particular H-2 haplotype, while less than 0.01% appear to be specific for minor histocompatibility antigens (*Bevan* et al., 1976).

3.3.7.2.4. Why H-2K and H-2D in the Mouse? One of the puzzling features of H-2 restriction has been the clonal distribution of anti-H-2K and H-2D receptors on the T killer cell. Thus, not only are H-2K and H-2D distinctly specified, but the T killer population is virtually duplicated (quadruplicated in an F_1 H-2 heterozygote) with respect to a particular anti-X and made unique by the clonality of anti-H. Since every infected target cell in the body is expected to carry both H-2K and H-2D antigens, there seems to be no need of separate T killer cells on this basis. However, if we consider the genetic origin of anti-X and anti-H recognition structures, an intriguing possibility arises.

During differentiation of stem cells to effector T cells there is a selection for cells displaying an anti-H receptor with specificity for self H; it is relevant to note that tolerance experiments (*Zinkernagel,* 1976; *von Boehmer* and *Haas,* 1976; *Pfizenmaier* et al., 1976) show that the "self" category in H-2 restriction includes H-2 antigens by which tolerance has been acquired. Following a selection of anti-H on the basis of "private" specificities of H-2, the somatic variation process generates an array of variants from the unused remainder of the germ-line pool. In other words, a T-cell precursor would display a receptor in the anti-H position and be subject to selection for antiself H. The T cells at this stage have either anti-H-2K *or* anti-H-2D in the anti-H position, and the germ-line genes coding for these receptors are locked into fixed expression. After this selection, a somatic variation mechanism operates on residual unused germ-line genes to generate the anti-X repertoire.

Consider the case of a particular specificity of anti-X (say X′) which arises only by the mutation of a particular germ-line gene (say anti-H-2K^d). In a mouse which is H-2^d, the T cells with anti-H-2K^d in the anti-H position cannot generate the anti-X′ specificity. However, the T cells with anti-H-2D^d will be able to generate anti-X′ from the unused anti-H-2K^d germ-line gene. If the X′ antigen were associated with some deadly pathogen, H-2^d mice would survive by virtue of the clonal distribution of anti-H-2K and anti-H-2D receptors; thus H-2K and H-2D genes with their distinct antigenic specificities provide a potentially important component in the overall functioning of a successful immune system.

There are some experimental results which would be consistent with this interpretation. Perhaps the best example is in the TNP system used by *Shearer* and collaborators where, if a particular allele of H-2D is the only H-2 antigen shared between the killer and TNP target, there is no effective lysis. Substitution of NNP for TNP as the non-H-2 antigen, while keeping everything else the same, allows target cell lysis to occur. The gene controling unresponsiveness was mapped in the Ir-1 region of the H-2 gene complex (*Schmitt-Verhulst* et al., 1976). Within the framework of H-2 restriction proposed here, this Ir-1 control of H-2 restricted killing represents the first clear evidence in favor of the genes coding for the T-cell receptor located in the Ir-1 region.

Epilog

The phenomenon of H-2 restriction presents a major challenge to current modes of thinking in cellular immunology, and it seems likely that the resolution of this enigma will provide insights as profound as the clonal selection theory did in confronting the antibody problem. Although this chapter has attempted to comprehensively evaluate the H-2 T killer phenomena and has proposed some rather radical views, the result has been another piecemeal effort in the larger problem – that of understanding the immune system. There has been no shortage of theories that account for one or another of the multiplicity of phenomena which fall within the domain of immunology, but as with numerous other areas of biology, we do not at present have the conceptual framework with which to appreciate the complexity of biologic systems. We are in the position of having a vast amount of data but very few ways of dealing with it.

In this essay I have taken a particular aspect of the immune system and tried to distill the essential rules which are needed to account for the experimental observations. The rules that emerged appeared to be very arbitrary when viewed against current concepts of cellular immunology; rather than discard the rules, I have attempted to draw in the rough outlines of a new concept of the immune system. By my taking an approach which is essentially that of Darwinian evolution and applying this mode of thinking to a component of the individual, the immune system, there has emerged a series of concepts that are compatible with the rules of H-2 restriction and do not obviously contradict other well-documented immune phenomena.

Perhaps the most difficult aspect of H-2 restriction is the need to answer the questions why H-2, why self H-2, why H-2K and H-2D, and why do they only kill allo-H-2. It became clear that the central issue was why self H-2 and not self "something else." The T-cell receptor cannot be expected to "know" self H-2 a priori; the knowledge must emerge via some selective or learning process in somewhat the same way as the broader distinction between self and nonself must be learned. In order to learn specificity, it is clearly necessary that the T-cell receptor be able to recognize H-2 antigens as such and, similarly, recognize X antigens. However, even if two receptors are accepted as the specific recognition structures for self H-2 and nonself-X, this cannot account for the kind of specificity observed in allogenic killing, where allo-H-2 antigens are the only class of nonself antigens that do not have to be associated with self H-2. In other words, the importance of H-2 in the effector reaction of killer T cells transcends antigenic composition, and the rule had to be introduced that the killing signal could only be delivered successfully at a restricted site which was intimately associated with the H-2 molecule on the cell surface. Discussion of H-2 restriction is thus a two-part problem – one part deals with restriction in the broad sense and the question why H-2, and the second part is concerned with the specificity elements, such as how anti-H-2 self is learned and why such a high frequency of allo H-2 reactive T killers.

This essay has not gone far toward rationalizing the part about H-2 in general; this has been tacitly taken as an assumption. On the more precise immunologic question of specificity, I have tried to give a detailed rationale which reduces to an analysis of selection mechanisms, for self H-2 versus self non-H and self-nonself discrimination. This analysis was based on the principle of an immune system arising to combat infectious agents, intracellular parasites in particular. The first major break with tradition came when the killing process was directed to effect a self-nonself discrimination – self reactive T cells autodestruct while nonself T cells survive. However, this break was with the mechanistic process which is usually considered an intracellular signaling event, but by emphasizing the importance of clonal elimination of self-reactive cells in the thymus, we return to *Burnet's* original postulates. Extending the principles of selection based on temporal and positional differences that were used in the self-nonself discrimination process, it was possible to devise a means of selecting self H-2 antigen from self non-H antigens. However, the diversity of anti-H-2 receptors is considerably less than the diversity of anti-X receptors, and it would have been unreasonable to eliminate the vast majority of cells with anti-non-H specificity in the anti-H selection process. It is perhaps conceivable to envisage a special set of genes coding for anti-species H and another set for anti-X. The conservative alternative presented here is that there is one pool of V-genes but two C-genes, one associated with the anti-H and the other the anti-X functions. Providing there is somatic mutation of a limited number of germ-line genes to give the anti-X series, as seems the case with the immunoglobulin series, this mutation process should be controlled so that (1) mutation is not expressed until after anti-H self has been selected from the germ-line and (2) mutation is at a sufficiently low rate that many, but not all germ-line sequences are altered. This latter condition allows anti-X to frequently have nonself H specificity as present in the germ-line and thus accounts for a relatively high frequency of allo-H-reactive T cells in comparison with the frequency of non-H-reactive, i.e., antiviral for example.

As *Klein* (1975) noted, there is an exceptionally high degree of polymorphism in the species alleles of H-2K and H-2D; a similar situation is apparent in the human H-2 equivalent, i.e., HLA antigens. Numerous attempts have been made to rationalize this unusual degree of polymorphism (*Bodmer,* 1972; *Burnet,* 1973; *Bodmer,* 1973) including some based on extensions of the H-2 restriction effect (*Terhorst* et al., 1976; *Bridgen* et al., 1976). Amino acid sequence data from several laboratories for H-2 (*Henning* et al., 1976; *Silver* and *Hood,* 1976; *Vitetta* et al., 1976) and HLA (*Terhorst* et al., 1976; *Bridgen* et al., 1976) has shown that in the first 30 residues from the NH_2 terminus there is 30% sequence variation between H-2 antigens whether the antigens are alleles of H-2K, H-2D antigens chosen at random, or of the same haplotype; there appear to be conserved, species-specific residues as well as a few invariant residues common to HLA and H-2. The degree of sequence variation in H-2 antigens is of the same order as immunoglobulins, at least so far as the first 30 residues of H-2 are representative of the whole; needless to say, such variability in H-2 and HLA provides ample material for endless speculation. In this essay I have not taken issue with the polymorphism of H-2, and in part this has been because there seemed no essential need to have a polymorphic H system. However, when considering the concept of germ-line V genes that are maintained to code antibody specificities directed against the species H allelic antigens, there is clearly an advantage to having many allelic antigens and, thus, many germ-line V genes. The polymorphism of H-2 is then a secondary effect and reflects the situation in which any H-2 antigenic determinant is acceptable so long as there is a corresponding V gene that can recognize it. Spontaneous mutations are continually occurring and being selected on the basis of successful function. If the selection pressure which determines permissable H-2 antigenic specificity arises primarily from the polygenic V gene pool, then the polymorphism of H-2 will be a direct reflection of the polygenism of the V genes. Therefore I regard the polymorphism of H-2 as a second-order consequence of having a pool of genes that are selected and maintained in the germ-line because they have specificity for H antigens of the species; H antigens are important, because they are intimately associated with the delivery of killing signals.

Having made these rules and rationalizations on the basis of T killer cell function and the H-2 restriction effect, there follows the natural question – what about other T-cell functions, such as suppression and cooperation? It is difficult to resist the temptation to transpose the ideas that have emerged from the H-2 restriction to Ia or Ir restriction. However, T killer function is very different from T cooperator function, and yet, despite the vast volumes of data on T-B collaboration, the phenomenon remains less clearly documented than T killing. Rather than attempt here a fragmentary and cryptic commentary of T-B collaboration based on the same approach as was taken for H-2 restriction, it seems more profitable to continue the evolutionary analysis developed here as a way of rationalizing the various rules of H-2 restriction and see how such a conceptual approach can point the way through the jungle of data. Considering the magnitude of such a journey, it is best left for another occasion.

One of the more significant outcomes of this essay to my mind is the realization that the immune system can be successfully treated as an evolutionary unit which contributes to survival by combating infectious diseases. Although there may be those who disagree with my argument that the immune system is primarily a survival kit which evolved to meet the challenge of parasitic invasion, the principle of evolution and selection operating to produce an immune system seem worthy of further analysis. One further point not emphasized here is that what we call an immune system is probably simply the vertebrate solution to infection for, to take an extreme view, bacteria also cope with viral infection, and they certainly do not have what we would call an immune system. Considerable attention has been given to invertebrate immunity and the question of self-nonself discrimination; however, there are several distinct aspects of self, such as surface recognition, organ recognition, even psychic recognition, and most of these do not obviously fall within the domain of immunology. There are multiple mechanisms of resistance to disease of which vertebrate immunity is only one example. Perhaps by paying closer attention to what is being recognized rather than recognition per se, we can draw more useful distinctions.

Acknowledgements. In closing – my confessions: The origin of this essay is in the work and thoughts of others. Most outstanding among the many are Drs. M. Cohn, M. Bevan, R. Blanden, and R. Zinkernagel. To them I owe thanks for sharing their excitement and wisdom. However, in adding my own biases and prejudices, without giving them the rights of censorship, I accept responsibility for the errors and inadequacies and give them credit for the remainder.

An apology is due for all those whose contributions have not been formally acknowledged. I have been deliberately selective in compiling a short bibliography, thus giving the novice a less awesome introduction to the mysteries of cellular immunology.

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Central α-Adrenergic Systems as Targets for Hypotensive Drugs*

WALTER KOBINGER**

Contents

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* This paper is dedicated to the memory of Franz Theodor v. Brücke (1908-1970), Professor of Pharmacology at the University of Vienna from 1948 to 1970.

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1. Introduction

This survey deals mainly with the mode of action of two well-known antihypertensive drugs, clonidine and α-methyldopa. The fact that substances which stimulate α-adrenoceptors are able to lower the blood pressure was surprising for many researchers as it complicated the interpretation of many experimental results and contained an element contradictory to classical pharmacologic thinking. This situation may be best illustrated by the story of the discovery of the hypotensive action of clonidine. This drug was originally synthesized by *H. Stähle* (1966) in order to obtain, on the basis of its imidazoline structure, a vasoconstricting and decongesting agent. Clonidine showed the expected vasoconstricting action but when *M. Wolf,* a physician of the trial group of a pharmaceutical company, carried out the first human studies (by dropping a few mg of this drug into the noses of his secretary and himself) he immediately discovered not only the sedative, but also the hypotensive and bradycardic effects of this drug (*Graubner* and *Wolf,* 1966; *Wolf,* personal communication). This observation gave rise to a great number of investigations which are documented in more than 1000 original papers on clonidine (up to July 1976).

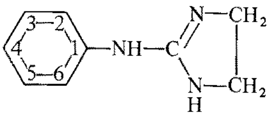
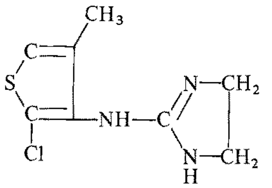
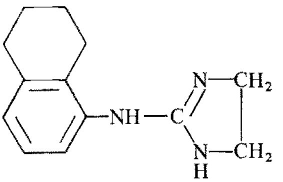
A number of compounds will be discussed which are structurally related to clonidine, and which have two pharmacologic properties in common with the latter drug: a direct stimulant effect on peripheral α-adrenoceptors and a hypotensive effect. Most of these compounds lower the blood pressure by a central action. On the basis of this pharmacologic pattern, these substances will be called "clonidine-like hypotensive drugs" (Tables 1 and 3). In the future, detailed pharmacologic studies may reveal differences between one or the other of these drugs and clonidine itself. The reviewer has endeavored to collect the properties common to the respective compounds and he believes that differences between them are mostly of a quantitative nature.

The main purpose of this presentation is to show that stimulation of certain central α-adrenoceptors causes hypotension; until now this was best documented for clonidine. However, many questions concerning central and peripheral adrenoceptors and cardiovascular actions are still open, and current concepts may require modification in the future. Part of this review has been devoted to a description of the peripheral pharmacologic effects of clonidine-like hypotensive agents as a basis for the understanding of more complicated processes in the CNS. For the purpose of comparison, some other imidazolines which exert α-adrenergic effects at peripheral sites, but do not decrease the blood pressure after systemic injection, will also be discussed (Tables 2 and 4).

α-Methyldopa had been in clinical use for some years before its central mode of action was revealed; the drug requires metabolic conversion to α-methyldopamine and α-methylnoradrenaline for its effect, and its cardiovascular effects are not easily demonstrable in acute animal experiments. The direct action and the immediate effect of clonidine-like drugs may explain why these agents are the preferred tools for investigations of central α-adrenoceptors. The peripheral pharmacologic effects of α-methylnoradrenaline, the active metabolite of α-methyldopa, have been reviewed by *Holtz* and *Palm* (1966).

A number of published symposia have been devoted to the question of central nervous blood pressure regulation (edited by *Davies* and *Reid,* 1975; *Milliez* and *Safar,* 1975; *Struyker Boudier* et al., 1975b; *Julius* and *Esler,* 1976; *Onesti* et al., 1976). The central hypotensive actions of α-methyldopa and clonidine were reviewed by *Henning* (1969a), *Schmitt* (1971), *Kobinger* (1973, 1975) and *van Zwieten* (1975a).

Table 1. Chemical structures of clonidine-like hypotensive drugs

Structure	Name, Code	R[a]
a) Aminoimidazolines ⟷	Iminoimidazolidines[b]	
	Clonidine, St 155 Catapres	2,6-$(\mathrm{Cl})_2$
	St 93	2-Cl, 6-Me
	St 95, Ba 3091	2, 6-$(\mathrm{Me})_2$
	Tolonidine, St 375	2-Cl, 4-Me
	St 363	2, 4-$(\mathrm{Cl})_2$
	Flutonidine, St 600	2-Me, 5-F
	St 608	2-Cl, 3-Me
	St 1697	2-Et, 6-Me
	Thiamenidine, Hoe 440	
	Tramazoline Rhinospray	

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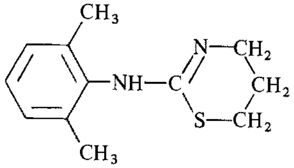
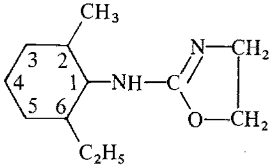
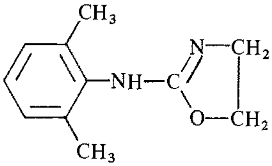
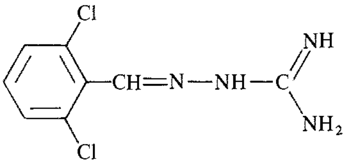
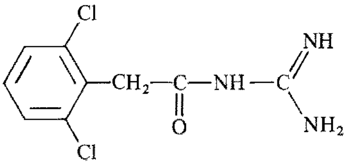
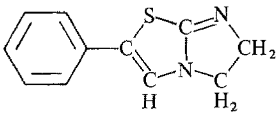
[a] Me, methyl; Et, ethyl.

[b] Because of the possibility of tautomeric forms, a shift of the C=N double bond towards the bridge N results in the respective imino form (see Fig. 1).

Table 1 (continued)

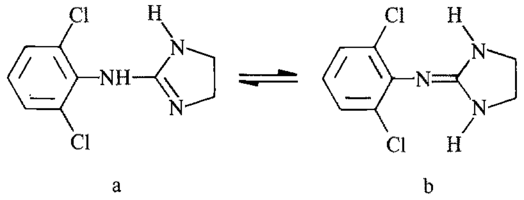
Structure ⟷	Name, Code
b) Amino-Dihydrothiazines	Imino-Tetrahydrothiazines[b]
	Xylazine, Bay 1470 Rompune
c) Aminooxazolines ⟷	Iminooxazolidines [b]
	Bay a 6781
	LD 2855
d) Guanidines	
	Guanabenz, Wy 8678
	BS 100-141
e) Bicyclic ring system	
	# 44.549/1

2. Clonidine-like Drugs

2.1. Chemistry

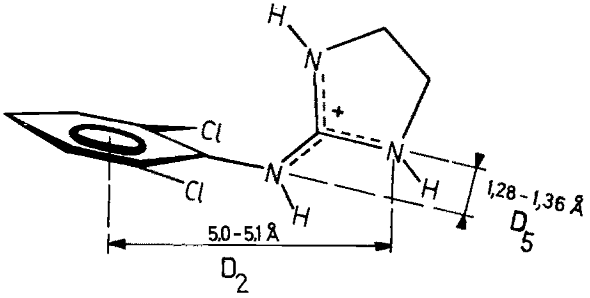
The synthesis of clonidine was described by *Stähle* and *Pook* (1971). The chemical structures of the two possible tautomeric forms of the clonidine base are shown in Figure 1. Spectral studies (UV, IR, NMR) and the use of methylated analogues of clonidine have shown that the 2-(arylimino)-imidazolidine tautomer (Fig. 1b) is the predominant form (*Jen* et al., 1972; *Stähle* and *Pook,* 1972; *Pook* et al., 1974).

Fig. 1a and b. Chemical structure of clonidine base in two possible tautomeric forms: 2-(2,6-dichlorophenylamino)-2-imidazoline (a) and 2-(2,6-dichlorophenylimino)-2-imidazolidine (b). The latter is predominant



Clonidine has a pk value of 8.05 (*Struyker Boudier* et al., 1974b; *Rouot* et al., 1976) so it is present in body fluids of pH 7.4 mainly (ca 85%) in the protonated form (Fig. 2). As indicated in Figure 2, the two ring systems are not on the same level but are aplanar (*Rouot* et al., 1973). Both rings have an angle of 34°, indicating a nonperpendicular structure (*Meerman-van Benthem* et al., 1975). The distance (D_2) between the center of the aromatic ring and one nitrogen of the imidazoline ring was calculated to be 5.0 - 5.1 Å and the distance (D 5) between the nitrogen and the plane of the benzene nucleus was calculated to be 1.28 - 1.36 Å (*Wermuth* et al., 1973). Both numbers fit a model of the α-adrenoceptor derived from measurements of interatomic distances in a series of phenylethylamines (*Pullmann* et al., 1972). A further description of the ground-state geometry of clonidine base has been given by *Meerman-van Benthem* et al. (1975). Physico-chemical data on clonidine and related compounds has been provided by *Struyker Boudier* et al. (1974b), *Hoefke* et al. (1975) and *Rouot* et al. (1976) (see also Section 4.3.).

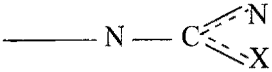
Fig. 2. Clonidine in protonated form. Note the aplanar conformation of the two ring systems, and the distances D_2 and D_5 (according to *Wermuth* et al., 1974)



The class of "clonidine-like hypotensive drugs" in Table 1 contains a number of different chemical groups but all agents exert direct stimulation of peripheral α-adrenoceptors and a hypotensive effect of central nervous origin. Obviously this list will be enlarged in the future by a great number of compounds but in this review only those which fulfill both criteria are considered. This limitation seems necessary because small changes in the chemical structure may shift the mechanism of the hypotensive action from "clonidine-like" to one of a peripheral type of adrenergic neurone blockade or adrenoceptor blockade (*Jen* et al., 1972; *Bream* et al., 1975).

Table 3 gives a survey of the literature reporting "clonidine-like" effects. In some studies, bradycardia was accepted instead of hypotension. However, care was taken to exclude reflex bradycardia as a response to elevated blood pressure.

In spite of the great variety of chemical structures in Table 1, all compounds contain, as a common structure, an "amidine moiety" (*Jen* et al., 1972; 1975):



where x = C, N, O or S. This includes cyclic guanidines, cyclic isoureas and isothioureas as well as open-chain guanidines.

Tables 2 and 4 show some imidazolines with peripheral α-adrenoceptor stimulating properties, but without hypotensive action following systemic administration (i.v., subcutaneous or oral) in contrast to intracerebral administration (see Section 2.4.2.1.). Some of these imidazolines are closely related chemically to clonidine. Various attempts have been made to correlate chemical structure, physicochemical parameters and central hypotensive activity in imidazoline derivatives (*Kobinger,* 1974; *Stähle,* 1974; *Struyker Boudier* et al., 1974b; 1975a; *Hoefke* et al., 1975; *Rouot* et al., 1976). Good results were recently obtained with a number of substances structurally related to clonidine (*Timmermans,* 1976).

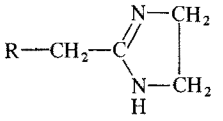
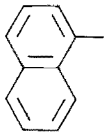
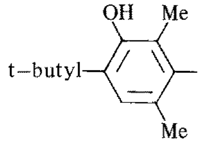
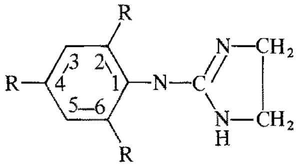
Addendum during print: Another group of typical clonidine-like substances has been found recently: heterocyclic oxazoloazepines. (*Kobinger* and *Pichler,* 1977). It remains open for the future, whether these structures also fit into the α-adrenoceptor model, as described above.

2.2. Peripheral Effects

2.2.1. Stimulation of Peripheral α-Adrenoceptors

Sympathomimetic effects have been observed for clonidine and clonidine-like substances using various methods (see Tables 3 and 5). The α-adrenergic

Table 2. Imidazolines lacking hypotensive effect following systemic administration

Structure	Name, Code	R[a]
	Naphazoline Privin	
	Oxymetazoline Nasivin	
Clonidine derivatives[b] 	St 91	2,6 – $(Et)_2$
	St 666	2,6 – $(Cl)_2$, 4-OH

[a] Me, methyl; Et, ethyl.

[b] Because of the possibility of tautomeric forms, a shift of the C=N double bond towards the bridge N results in the respective imino form.

nature of these effects was evident from the antagonism by α-adrenoceptor blocking agents (*Kobinger* and *Walland*, 1967a; *Boissier* et al., 1968). An indirect sympathomimetic action was excluded after experiments involving the depletion of endogenous noradrenaline stores by pretreatment with reserpine and α-methyl-p-tyrosine (an inhibitor of tyrosine hydroxylase; *Kobinger* and *Walland*, 1967a; *Boissier* et al., 1968; *Kobinger* and *Pichler*, 1974, 1975a). From the results, it was concluded that clonidine and related drugs directly stimulate peripheral α-adrenoceptors. Stimulation of vascular α-adrenoceptors, by direct injection of clonidine or clonidine-like drugs into the arteries of various preparations, caused vasoconstriction and a decrease in regional blood flow (*Kobinger* and *Walland*, 1967a; *Constantine* and *McShane*, 1968). After rapid i.v. injection of clonidine-like drugs into intact animals, a transient increase in blood pressure (due to stimulation of vascular α-adrenoceptors) typically precedes the hypotensive phase (see Fig. 3; *Kobinger* and *Walland*, 1967a).

A specific, direct stimulation of α-adrenoceptors by naphazoline and oxymetazoline was described in 1965 by *Mujic* and *van Rossum*. This ob-

Table 3. Clonidine-like hypotensive drugs. The third column indicates the references for hypotensive and/or bradycardic effects. The fourth column indicates the methods and the fifth column the references for the α-adrenergic effects of the substances

	Substance	Hypotension -bradycardia references	α-Adrenergic effect	
			Method	References
Imadazolines	Clonidine, St 155	14	a, b, c, d, e, f, g	1, 3, 11, 14
	St 93	1	a, b, c	1, 8
	St 95	2	a, c	15
	Tolonidine, St 375	1, 12	a, b, c	1, 12
	St 363	2	a	2
	Flutonidine, St 600	1, 3	a, b, c, e	1, 3, 8
	St 608	1	a, b, c	1
	St 1697	2	a	2
	Thiamenidine	7	c	7
	Tramazoline	3	c, e	3, 8
Thiazines	Xylazine, Bay 1470	5	a, c, d	5, 1, 11
Oxazolines	Bay a 6781	13	c	13
	LD 2855	11	a	11
Guanidines	Guanabenz Wy, 8679	6	c	6
	BS 100 141	4	a, c, g	4
	#44549	10	a	10

a) hypertension in spinal or pithed animals
b) mydriasis, rats
c) initial pressor effect
d) contraction, nictitating membrane
e) isolated intestine, inhibition of movements
f) contraction, isolated vas deferens, rat
g) contraction, isolated vascular strips

1. *Hoefke* et al. (1975); 2. *Kobinger* and *Pichler* (1975c); 3. *Struyker Boudier* et al. (1974b); 4. *Scholtysik* et al. (1975); 5. *Kroneberg* et al. (1967); 6. *Baum* et al. (1970, 1976); 7. *Lindner* and *Kaiser* (1974); 8. *Struyker Boudier* et al. (1975a); 9. *Starke* et al. (1975); 10. *Boyajy* et al. (1972); *Van Zwieten* (1975b); 11. *Autret* et al. (1971); *Schmitt* and *Fénard* (1971); 12. *Cosnier* et al. (1975a; b); 13. *Jacobs* et al. (1972); 14. *Hoefke* and *Kobinger* (1966); 15. *Kobinger*, unpublished.

Table 4. Imidazolines lacking hypotensive effect following systemic administration. α-Adrenergic effects. For methods used and references see Table 3

Substance	α-Adrenergic effect	
	Method	References
Naphazoline	a, c, e, g	2, 8, 9, 11
Oxymetazoline	a, c, e, g	2, 8, 9
St 91	a, b, c, g	1, 2, 8
St 66	e	8

Table 5. α-Adrenergic potencies of various substances at different peripheral effectors

Substance	Blood pressure increase in rats		Isolated rabbit				Isolated rat vas deferens[j]		
	pithed[a]	spinal[b]	intestine[c]		pulmonary artery strip[d]				
	R	R	i.a.[e]	pD_2[f]	i.a.	pD_2	i.a.	pD_2	pA_2[k]
Clonidine-like drugs									
Clonidine	0.029	0.03	0.4	5.2	0.32 0.4[g]	6.4 6.2[g]	0.77 0.37[l]	2.79 4.52[l]	5.04
St 93		0.02	0.3	5.6					
Tolonidine		0.01							
St 363		0.002							
Flutonidine		0.002	0.5	5.4					
St 608		0.002							
St 1697		0.1							
Xylazine	0.003	0.001							
LD 2855	0.028								
BS 100 141					0.3[h] 0.8[i]	5.8[h] 6.2[i]			
Tramazoline			1.0	6.2	0.6	6.63			
Imidazolines without hypotensive action									
Naphazoline	0.087	0.15	1.0	6.1	0.65	6.92	0.6	3.3	5.5
Oxymetazoline	0.1	0.09	1.0	8.8	0.75	7.06	0.1	3.7	6.0
St 91		0.08	0.9	6.2					
St 666			0.3	5.4					
Phenylalkylamines									
Noradrenaline	1.0	1.0	1.0	6.8	1.0	7.25	1.0	5.2	
Adrenaline			1.0	7.1	1.0	7.90	1.1	5.3	
Methoxamine					1.0	5.57			
Phenylephrine			1.0	6.0	1.0	6.61	0.9	4.9	
α-methyl-noradrenaline			1.0	6.2	1.0	6.51			

[a] *Autret* et al. (1971); R = relative potency; [b] calculated from *Hoefke* et al. (1975); *Kobinger* and *Pichler* (1975c); [c] *Struyker Boudier* et al. (1975a); [d] *Starke* et al. (1974, 1975; [e] intrinsic activity; [f] agonistic affinity; [g] calculated from *Constantine* and *McShane* (1968), rabbit aortic strip; [h] *Scholtysik* et al. (1975), dog aortic strip; [i] *Scholtysik* et al. (1975), dog venous strip; [j] *Kobinger* and *Pichler* (1975c); [k] antagonistic affinity; [l] *Brugger* et al. (1969).

servation has been extended by various methods to the drugs St 91 and St 666 (Table 4). Systemic injections of these substances caused a profound and long-lasting increase in blood pressure and total peripheral resistance, and a reflex decrease in heart rate and cardiac output (*Autret* et al., 1971; *Hoefke* et al., 1975; *Kobinger* and *Pichler*, 1975c).

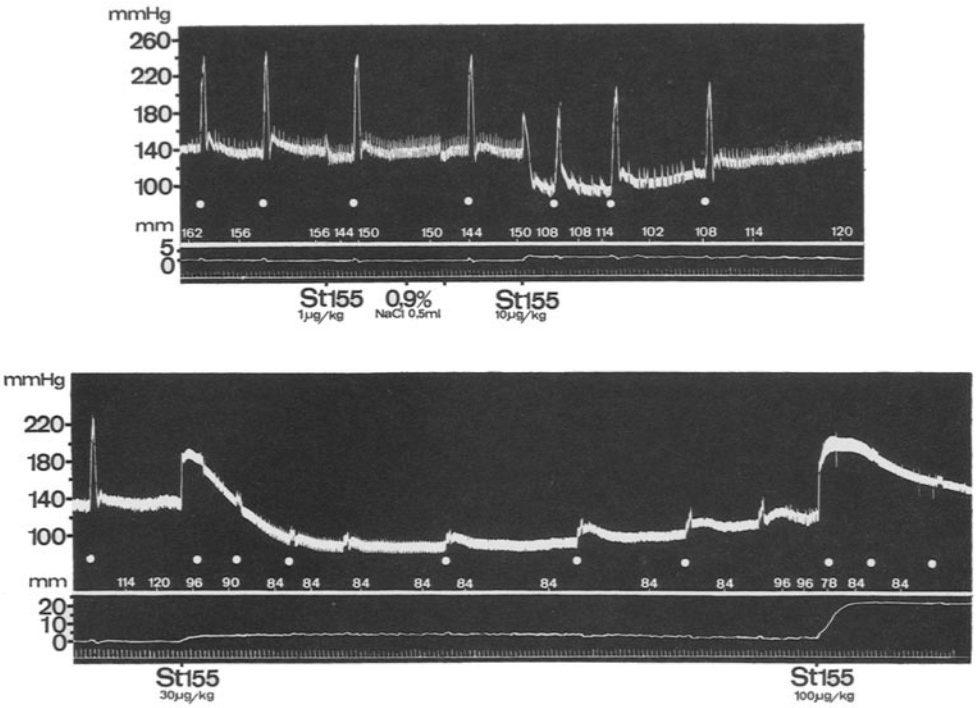


Fig. 3. Effect of i.v. injection of increasing doses of clonidine (St 155). (Anesthetized dog, both vagi cut). The upper trace shows blood pressure, the dots indicate where both carotid arteries were clamped for 1 min and the numbers are heart rate in beats/min. The lower trace shows tension developed by one nictitating membrane. The time signal occurs at 1 min intervals. Note: biphasic effect on blood pressure, decrease in heart rate and increase in nictitating membrane tension. From *Hoefke* and *Kobinger* (1966)

2.2.2. α-Adrenergic Potency and Intrinsic Activity at Peripheral Effectors

Table 5 reviews data on the α-adrenergic potency of clonidine-like substances, other imidazolines and some phenylalkylamines. When dose-response curves in isolated organs were analyzed, clonidine always exerted a maximal response that was smaller than the response to noradrenaline, (i.e. the intrinsic activity was less than 1.0). Clonidine is therefore characterized as a partial agonist or partial antagonist (*Ariëns,* 1964). This is in accordance with the α-adrenoceptor blocking effects of the drug which are found in various animal species (*Hoefke* and *Kobinger,* 1966; *Boissier* et al., 1968) and in various isolated systems and organs (*Bentley* and *Li,* 1968; *Constantine* and *Mc Shane,* 1968; *Coupar* and *Kirby,* 1972). An intrinsic activity smaller than unity was also reported for other clonidine-like drugs and for imidazolines lacking hypotensive action. However, there were differences between the substances with respect to the test organs (Table 5). The most striking example is oxymetazoline, with an intrinsic activity of 1.0 on the isolated intestine of the rabbit, 0.75 on the pulmonary artery strip of the same species and 0.1 on the vas deferens of the rat. In the latter preparation, oxymetazoline was a noradrenaline antagonist nearly as potent as phentolamine which had a pA_2 value of 6.5. The relative potencies (R) and the affinities to α-adrenoceptors (pD_2 values) in Table 5 also reveal considerable differences between the sympathomimetic drugs in different organ preparations. Such differences may be explained by different types of α-adrenoceptors in different organs as suggested by *van Rossum* (1965) on the basis of results with agonists and antagonists. These results should be kept in mind when considering the differences between central and peripheral α-adrenoceptors (Section 4.3.).

2.2.3. Decreased Release of Noradrenaline by Stimulation of Presynaptic α-Adrenoceptors

Under certain experimental conditions, clonidine decreases the response of effector organs to electric stimulation of their sympathetic nerves. As shown in Figure 4 this inhibition appears only with high doses of clonidine and at low stimulation frequencies. In analogous experiments, bretylium and guanethidine decreased responses over a wide range of stimulation frequencies, thus differentiating the effect of "specific" adrenergic neurone blocking agents from those of clonidine (Fig. 4; *Kobinger*, 1967). In isolated perfused rabbit hearts, *Starke* and *Schümann* (1971) and *Werner* et al. (1972) showed that clonidine diminished the outflow of noradrenaline into the per-

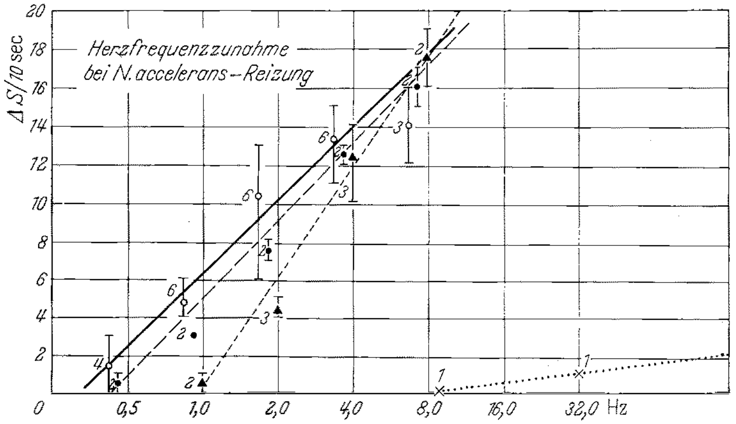


Fig. 4. Relation between frequency of electric stimulation of right cardiac nerve and increase in heart rate. (Anesthetized cat). Abscissa: stimulation frequency (square wave impulses, 5 V, 2 msec, trains of 25 sec duration). Ordinate: mean increase in beats / 10 sec with range. The numbers near each point = n of stimulations. ○ Controls; ● clonidine, 30 μg/kg; ▲ clonidine 100 μg/kg; x guanethidine, 3.2 mg/kg (all substances i.v.). Note: the higher dose of clonidine decreases the response, but only at lower stimulation rates. From *Kobinger* (1967)

fusion fluid after electric stimulation of the sympathetic cardiac nerve. This inhibition was seen at low but not at high stimulation frequencies. During the following few years, work in several laboratories led to the concept that peripheral and central noradrenergic nerve endings contain (presynaptic, prejunctional) α-adrenoceptors which mediate a negative feedback control of transmitter release from the nerve ending (see *Langer* et al., 1975; *Rand* et al., 1975; *Starke* et al., 1975). Activation of the receptor leads to reduction, and blockade to facilitation, of the secretion of noradrenaline per orthodromic nerve impulse. This area has recently been reviewed extensively by *Starke* (1977).

In accordance with this hypothesis, various clonidine-like substances, imidazolines without hypotensive effect, and some phenylalkylamines have been shown to decrease organ response and/or noradrenaline release at low frequency stimulation (*Baum* et al., 1970; *Deck* et al., 1971; *Werner* et al., 1972; *Boyajy* et al., 1972; *Starke,* 1972; *Vizi* et al., 1973; *Lindner* and *Kaiser,* 1974; *Scholtysik* et al., 1975). For a number of sympathomimetic drugs, *Starke* and colleagues (1974; 1975) found different potencies at pre- and post-synaptic α-adrenoceptors. These authors used isolated pulmonary artery strips of rabbits to study the myogenic contraction (postsynaptic response) and the change in tritium overflow (presynaptic response) after preincubation of the strips with [^{3}H]-noradrenaline. Transmural electrical stimulation was applied at 2 Hz. Equieffective concentrations were calculated from concentration-response curves. The drugs were classified into three groups: *group 1 (preferentially post-synaptic agonists),* methoxamine and phenylephrine; *group 2 (similar pre- and postsynaptic potencies)* noradrenaline, adrenaline and naphazoline; *group 3 (preferentially presynaptic agonists)* clonidine, oxymetazoline, α-methylnoradrenaline and tramazoline.

Variations in relative pre- and postsynaptic potencies of nearly 500-fold were found and explained by structural differences between the pre- and postsynaptic α-adrenoceptors, thus endowing certain agonists with selective affinities (*Starke* et al., 1975). Clonidine inhibition of presynaptic α-adrenoceptors should also be considered in some organs, since the drug increased contractions of guinea pig vas deferens in situ (*Kobinger,* 1967) and enhanced the release of noradrenaline from this organ in vitro (*Stjärne,* 1975).

Apparently, similar differences exist between pre- and post-synaptic α-adrenoceptors as exist between the postsynaptic α-adrenoceptors of different peripheral organs (see Section 2.2.2.). Thus, the post-synaptic cell response will not only depend upon the concentration of the drug in the biophase, but also on the relative pre- and postsynaptic affinity of the given drug with respect to agonistic as well as antagonistic action. Furthermore, the response will depend upon the prevailing tonic activity (i.e., frequency of orthodromic nerve activity) of the adrenergic nerve. The possible contribution of these peripheral effects to the hypotensive action of clonidine-like drugs will be discussed in Section 2.4.1.

2.2.4. Inhibition of Cholinergic Neurones

Paton und *Vizi* (1969) reported that noradrenaline and other sympathomimetic amines reduced the output of acetylcholine during electric stimulation. The effect of the drugs was dependent on the stimulation frequency, was antagonized by α-adrenoceptor antagonists and was, therefore, explained by effects on presynaptic receptors. Similarly, xylazine, clonidine, naphazoline and other chemically related sympathomimetic drugs inhibited the release of acetylcholine and the response to cholinergic nerve stimulation (*Kroneberg* et al., 1967; *Deck* et al., 1971; *Starke,* 1972; *Werner* et al., 1972).

From Sections 2.2.3. and 2.2.4., it may be concluded that the inhibition of transmitter release in adrenergic and cholinergic nerves is a general ability of α-adrenergic stimulating drugs (including the natural transmitters, noradrenaline and adrenaline). The process is characterized by a dependency on the frequency of orthodromic stimulation and, therefore, results in a modulation, rather than in a block, of physiologic events. For the pharmacologist, it is interesting that a number of α-adrenergic stimulating drugs have potencies at these "modulatory" sites that differ from their potencies for other α-adrenergic effects.

2.2.5. Effect on β-Adrenoceptors, Other Peripheral Receptors and Local Anesthetic Effect

No indication of β-adrenoceptor stimulation was provided by experiments involving the intraarterial injection of clonidine. Vasodilation was never observed (see Section 2.2.1.). In most studies using isolated or in situ hearts, no positive inotropic or chronotropic effects were reported (see *Kobinger,* 1973). In the isolated perfused guinea pig heart, clonidine (~5 μg/ml) exerted a positive inotropic effect. This increase in contractility was not antagonized by toliprolol (β-adrenoceptor antagonist), phentolamine or pheniramine. It was, however, antagonized by burimamide (a histamine H_2 receptor blocking agent), suggesting an agonistic action of clonidine upon histamine H_2 receptors in the heart (*Csongrady* and *Kobinger,* 1974). A stimulation of gastric H_2 receptors by clonidine was reported by *Karppanen* and *Westermann* (1973).

Subcutaneous injections of clonidine revealed a local anesthetic effect to cutaneous stimuli. A 0.12% solution was equieffective with 0.21% procaine. The relative activity of clonidine was 1.75 times that of procaine (*Hoefke* and *Kobinger,* 1966). Clonidine decreased conduction in the isolated frog sciatic nerve as effectively as procaine and was much more effective than procaine as a surface anesthetic on rabbit cornea (*Starke* et al., 1972).

A local surface anesthetic effect for compound LD 2858 had been described by *Guidicelli* et al. in 1958. Local anesthetic properties were also reported for xylazine (*Kroneberg* et al., 1967) and thiamenidine (*Lindner* and *Kaiser,* 1974) but only for high and toxic concentrations of oxymetazoline and naphazoline (*Starke,* 1972).

The local anesthetic effect of these drugs should be considered when they are applied into the central nervous system in high concentrations.

2.3. Cardiovascular Depression

2.3.1. Cardiovascular Reaction Pattern

Rapid intravenous injection of clonidine (5-500 μg/kg) into anesthetized or conscious animals leads to a typical cardiovascular response pattern: an initial increase in blood pressure is followed by a gradual decrease; bradycardia and a decrease in cardiac output parallel the changes in blood pressure (Figs. 3 and 5a; *Hoefke* and *Kobinger,* 1966; *Kobinger* and *Walland,* 1967a; *Boissier* et al., 1968; *Constantine* and *McShane,* 1968). The extent and duration of the pressor phase can be correlated with the dose (Fig. 3) and is inversely related to the speed of injection. No pressor phase was observed after slow intravenous infusion (*Nayler* et al., 1968), and no hypertension followed oral ingestion of therapeutic doses by humans. The extent of the initial pressor effect is negatively correlated with the initial blood pressure. Therefore, clonidine-like drugs may be overlooked in a general screening procedure if high doses are injected into laboratory animals which have a low initial blood pressure i.e., the pressor phase may completely mask the hypotension. There is little doubt that the initial pressor effect and the increase in peripheral resistance are due to the direct stimulation of α-adrenoceptors in the peripheral vascular bed (see Section 2.2.1.).

The hypotension is due to a fall of both systolic and diastolic pressure by about the same degree (*Bentley* and *Li,* 1968). The total peripheral vascular resistance, calculated as "mean blood pressure/cardic output," is initially increased, later falling to control levels during the hypotensive phase (Fig. 5a). In the experiments depicted in Figure 5, the hypotension is solely due to the decrease in cardiac output. Similar results were obtained in animals by *Maxwell* (1969) and *Laubie* and *Schmitt* (1969), and in humans by *Grabner* et al. (1966) and *Vorburger* et al. (1968). However, there are also reports of a decrease in total peripheral resistance in animals (*Constantine* and *McShane,* 1968) and in humans (*Grabner* et al., 1966; *Muir* et al., 1969; *Onesti* et al., 1969). An important contribution to the question of whether this type of drug reduces blood pressure mainly by a decrease in cardiac output or by a decrease in total peripheral resistance (i.e., vasodila-

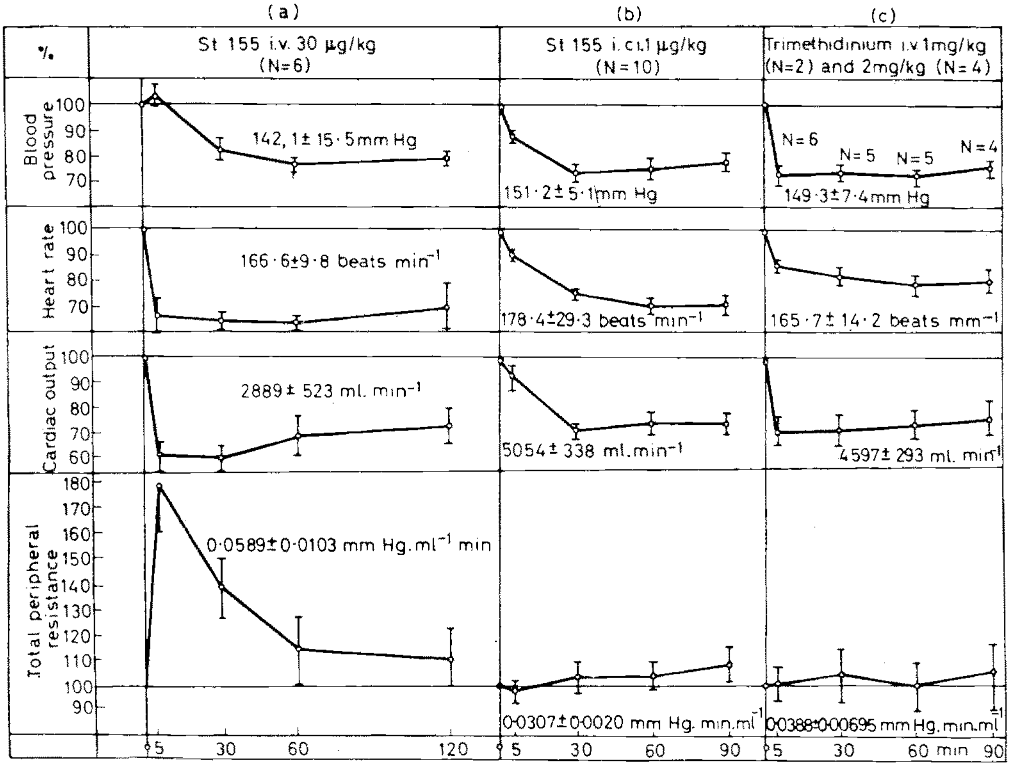


Fig. 5. Changes in cardiovascular parameters after injection of clonidine (St 155) or the ganglionic blocking agent trimethidinium. (Anesthetized dog). i.ci. = intracisternal injection (cisterna magna). Abscissa: time in minutes after injection of the drug. Ordinates: values in percent of control (mean ± S.E.M.). Numbers: absolute control values (mean ± S.E.M). Note the similar cardiovascular response pattern in all experiments. An initial increase in peripheral resistance is only seen after i.v. injection of clonidine. The cardiovascular parameters are lowered to approximately the same extent after 1 µg/kg, i.ci. and 30 µg/kg, i.v. From *Kobinger* and *Walland* (1967a, b)

tion) has been made by *Onesti* et al. (1971). Human subjects, in the supine position, responded to clonidine (300-450 µg, orally) with hypotension and a decrease in cardiac output, but with no change in total peripheral vascular resistance. However, in the upright position, in addition to a fall in cardiac output, a significant decrease in resistance was measured. A similar result had been obtained earlier with two other sympathoinhibitory drugs, guanethidine and α-methyldopa (*Chamberlain* and *Howard,* 1964). The state of the sympathetic tone in various parts of the cardiovascular system seems to determine whether these drugs preferentially decrease the sympathetic drive to the heart or to resistance vessels. Obviously, in the vertical position, the higher sympathetic drive in the peripheral vascular bed makes the resistance vessels an important site of action of these drugs.

Different vascular areas might be affected by clonidine-like drugs in different ways. In dogs with the heart and lung replaced by an oxygenator-pump system (heart-lung bypass), 5 μg clonidine /kg decreased blood pressure, but the increase in blood flow differed for various vascular beds (*Nayler* et al., 1966). *Merguet* and *Bock* (1973) measured blood flow in different vascular beds of humans using a heat clearance device. Intravenous injection of 150 μg clonidine decreased blood pressure, markedly decreased the blood flow in the skin, but increased blood flow in the calf muscles. These variations might be explained by differences in sympathetic tone and, in addition, by a different distribution of peripheral vascular α-adrenergic receptors in various vascular sections.

The typical pattern of an initial increase in blood pressure followed by long-lasting hypotension and bradycardia was reported for all clonidine-like drugs listed in Table 3 (see Table 3 for references). More detailed studies on various cardiovascular parameters were reported for the following drugs: tolonidine (*Cosnier* et al., 1975a,b; *Hoefke* et al., 1975), flutonidine (*Hoefke* et al., 1975; *Kho* et al., 1975; *Djawan* et al., 1976), St 93, St 608 (*Hoefke* et al., 1975), thiamenidine (*Lindner* and *Kaiser,* 1974) xylazine (*Hiese* et al., 1971) and BS 100-141 (*Scholtysik* et al., 1975). All these drugs caused effects similar to those of clonidine.

2.3.2. Quantitative Comparison of Hypotensive and Bradycardic Effects

When evaluating the hypotensive potency of a clonidine-like drug, one has to consider that the observed pressure changes depend on both the hyper- and the hypotensive action of the given drug. Both components depend on the initial blood pressure and sympathetic activity. The negative correlation between the pre-drug blood pressure and the pressor effect of these drugs has been mentioned above. In addition, there is a positive correlation between initial blood pressure and the hypotensive action; i.e., the higher the initial pressure, the greater the pressure decrease by the drug (*Kündig* et al., 1967). The complicated interaction between pressor and depressor effects of clonidine was recently illustrated by a study by *Wing* et al. (1975) in human patients. There was a correlation between plasma drug concentration and hypotensive effect only at lower plasma levels. At higher drug concentrations, the observed hypotensive effect was considerably smaller than expected, probably due to the increasing influence of the pressor component. Thus, quantitative comparisons of these drugs should only be done under standardized conditions. The following survey of published data, therefore, provides only a rough orientation. Dose-response curves for the hypotensive effect of clonidine have been obtained in various species (in parentheses are the doses in μg/kg, i.v., which gave an approximately 20-30 mm Hg decrease in blood pressure): rabbit (15; *Hoefke* and *Kobinger,* 1966), dog (10; *Nayler* et al., 1968) and rat (10; *Toda* et al., 1969). Guanabenz was injected i.v. into dogs and a dose of 100 μg/kg caused an approximately 20-30 mm Hg decrease in blood pressure (*Baum* et al., 1970). Thiamenidine (40 μg/kg, i.v.) was less effective in dogs than the same dose of clonidine (*Lindner* and *Kaiser,* 1974). For BS 100-41, no significant fall in blood pressure was found in anesthetized dogs or cats (doses up to 700 μg/kg and 100 μg/kg, respectively), whereas in DOCA-NaCl-hypertensive rats the drug caused hypotension (1 mg/kg and more p.o.; *Scholtysik* et al., 1975). To overcome the difficulties of the quantitative measurement of hypotensive activity, the bradycardic effect of the clonidine-like substances can be used. Rats were vagotomized and treated with atropine to exclude vagal reglex bradycardia. The heart rate was then measured 30 min after i.v. injection of the required drug (*Hoefke* et al., 1975; *Kobinger* and *Pichler,* 1975a). Assuming that, in the lower hypotensive dose range, clonidine-like drugs exert no bradycardia by a direct action on the heart (see Section 2.4.1.), the responses are due to sympathoinhibition. With this method, linear log dose-response curves were obtained and the dose corresponding to a decrease of 50 heart beats/min (ED50) was calculated for each drug. ED50 values in μg/kg were as follows (in parentheses are the potencies relative to clonidine): clonidine, in two different series, 5 and 9.1 (1); xylazine, 115 (0.08); St 93, 95 (0.53); St 95, 22 (0.4); tolonidine, 300 (0.02); flutonidine, 300 (0.02); St 608, 62 (0.08); St 1697, 60 (0.09). The imidazolines naphazoline, oxymetazoline and St 91, which do not lower blood pressure, did not elicit any consistent bradycardic response in this test within a reasonable dose range.

2.4. Mechanism of Cardiovascular Depression

The long-lasting decrease in blood pressure, heart rate and cardiac output, in response to the systemic administration of clonidine, resembled the response pattern to those drugs which exert their antihypertensive effect by a decrease in sympathetic activity (see Fig. 5): ganglionic blocking agents, reserpine, guanethidine and bretylium (see *Kirkendall* and *Wilson,* 1962; *Sannerstedt* and *Conway,* 1970). Naturally, such mechanisms were investigated during early research on clonidine-like drugs.

2.4.1. Possible Involvement of Peripheral Mechanisms

No peripheral actions of clonidine were found which sufficiently explained the cardiovascular depression. This has been reviewed previously (*Kobinger,* 1973) and included investigations into α-and ß-adrenoceptor blocking actions, adrenergic neurone blocking actions, ganglionic blocking actions, peripheral myogenic vasodilatation and direct influence upon myocardial function (*Hoefke* and *Kobinger,* 1966; *Kobinger* and *Walland,* 1967a; *Kobinger,* 1967; *Boissier* et al., 1968; *Rand* and *Wilson,* 1968; *Nayler* et al., 1969).

The impairment of peripheral adrenergic transmission by clonidine and xylazine, through stimulation of presynaptic α-adrenoceptors in adrenergic nerves (Section 2.2.3.), was thought to contribute to the overall antiadrenergic and hypotensive effect of these drugs (*Kroneberg* et al., 1967; *Scriabine* et al., 1970; *Starke* and *Schümann,* 1971). The fact that the drugs were mainly acting at low physiologic stimulation frequencies (about 5 Hz or less) was in favour of these arguments. However, recent research has shown that there exist other sympathomimetic agents with a similar or higher acitvity than clonidine at presynaptic α-adrenoceptors (e.g., noradrenaline, adrenaline, naphazoline, oxymetazoline, α-methylnoradrenaline and tramazoline). Some of these drugs have an affinity ratio for pre- and postsynaptic α-adrenoceptors similar to that of clonidine (see Section 2.2.3.). It is reasonable to assume that, following systemic administration, most of these drugs easily gain access to the peripheral sympathetic nerve endings and stimulate pre- and postsynaptic α-adrenoceptors. Thus, although all the sympathomimetic drugs listed above have virtually the same chance to decrease peripheral adrenergic nerve function by stimulation of presynaptic α-adrenoceptors, only a few (namely those of the clonidine-type) exert the typical pattern of cardiovascular depression.

An increase in peripheral baroreceptor activity by clonidine (20-30 μg/kg) is manifested by an increase in the electric discharges in rabbit aortic nerve (*Aars,* 1972; *Korner* et al., 1974). This effect is abolished by α-adrenoceptor blocking agents, and may be explained by a stiffening of the aortic wall of baroreceptor regions as a result of the vasoconstricting effect of clonidine (see *Heymans* and *Neil,* 1958). The stimulation of baroreceptors may be a contributory factor to the resetting of the baroreceptor response by clonidine (see Section 2.4.2.4.). It cannot, however, be essential for the cardiovascular depressor effect of clonidine as the drug was fully active after the section of afferent "buffer nerves" (*Boissier* et al., 1968; *Schmitt* et al., 1968).

2.4.2. CNS Mechanism

Clonidine exerts a cardiovascular reaction pattern which indicates an inhibition of the sympathetic system, but does not exert adequate effects at peripheral sites. Therefore, an action of clonidine on the central nervous system was proposed (*Kobinger* and *Walland,* 1967a, *Boissier* et al., 1968) and has been confirmed by subsequent investigations.

2.4.2.1. Administration of Drugs Into the CNS. In the first experiments, clonidine was injected into the cisterna cerebellomedullaris. The fate of drugs given by this route was illustrated by *Hamperl* and *Heller* (1933) who injected Chinese ink into cats and dogs. Within 1-2 min, ink particles were found in the fourth, third and lateral ventricles as well as in large areas of the subarachnoidal space.

Intracisternal injection of 1 μg clonidine/kg into vagatomized cats produced significantly greater hypotension and bradycardia than the same dose given i.v. (*Kobinger,* 1967). Intracisternal administration of an equieffective local anesthetic dose of procaine (2 μg/kg in 0.05 ml) was ineffective. After intracisternal injection of clonidine (1 μg/kg), an increase in spleen volume accompanied the hypotension. There were practically no indications of peripheral sympathomimetic effects on the nictitating membrane and pupil (*Kobinger* and *Walland,* 1967b). In dogs, the intracisternal injection of 1 μg clonidine/kg resulted in approximately the same decrease in blood pressure, heart rate and cardiac output as observed after i.v. injection of 30 μg/kg (Fig. 5; *Onesti* et al., 1971). The intravenous injection of 1 μg clonidine/kg was ineffective. Noradrenaline (5 μg/kg) is a more effective vasoconstrictor at peripheral sites but was ineffective when injected intracisternally (*Kobinger* and *Walland,* 1967b). Figure 5 shows that there was no initial increase in blood pressure and no change in total peripheral resistance after the intracisternal injection of clonidine in contrast to the i.v. injection of an equihypotensive dose.

Sattler and *van Zwieten* (1967) inserted a catheter through the distal part of the subclavian artery of cats until its tip remained just distal of the ostium of the vertebral artery. Retrograde injection results in high concentrations of a given drug in the lower brainstem. By this route, 0.25-2 μg clonidine/kg decreased blood pressure, while the same doses i.v. were ineffective. Similar results were obtained by the injection of clonidine into the vertebral artery of dogs. In addition, the drug decreased vascular resistance in the hindlimb (*Constantine* and *McShane,* 1968). *Sherman* et al. (1969) performed cross-circulation experiments in which the head and body of the recipient dogs had nerve connections only. Injection of 10 and 20 μg clonidine/kg into the donor's blood (which reached the recipient's head) lowered blood pressure and heart rate in the recipient's body.

Numerous reports have since been published, showing that clonidine decreased blood pressure and heart rate when injected into the cisternal spaces and the ventricular system of normal (*Schmitt* and *Schmitt,* 1969; *Onesti* et al., 1971; *Dollery* and *Reid,* 1973) and hypertensive animals (*Schmitt* and *Schmitt,* 1969; *Reid* et al., 1973). As these effects were obtained with low doses of the drug, they were taken as an indication for a central nervous site of action.

To localize the site of action more precisely, clonidine was administered to anatomically defined areas of the brain; this will be reported in Section 2.4.2.6.

Infusion of xylazine into the fourth cerebral ventricle of cats reduced the blood pressure more than i.v. infusion of the same dose (5 μg/kg/min for 10 minutes; *Heise* et al., 1971). Analogous results were obtained with injection of xylazine into the lateral ventricle of conscious hypertensive cats (*Finch,* 1974). In adrenalectomized cats, hypotension and bradycardia were produced by the injection of 3 μg of compound 44-549 into the vertebral artery (*Boyajy* et al., 1972). The hypotension was more pronounced than after i.v. injection of the same dose (*van Zwieten,* 1975b). BS 100-141 decreased heart rate and blood pressure in cats after the injection of 3 μg/kg into the lateral ventricle, the same dose i.v. was without effect (*Scholtysik* et al., 1975). A number of clonidine-like drugs (St 93, tolonidine, St 363, St 608) produced hypotension and bradycardia in vagotomized, atropine-treated cats when injected intracisternally in doses lower than those necessary for an effect after i.v. administration (*Hoefke* et al., 1975; *Kobinger* and *Pichler,* 1975c). All these drugs were less potent than clonidine. Thiamenidine, injected intracisternally into dogs (10-100 μg) or into the lateral ventricle of rats (2-16 μg) decreased the blood pressure (*Lindner* and *Kaiser,* 1974).

2.4.2.2. **Decrease in Electric Activity of Sympathetic Nerves.** Clonidine (3-30 μg/kg, i.v.) reduced or abolished the spontaneous electric discharges in preganglionic and postganglionic sympathetic nerve fibers (cervical sympathetic trunk, splanchnic nerve, inferior cardiac nerve, renal nerve) of cats (Fig. 6), dogs and rats (*Schmitt* et al., 1967; 1968; *Hukuhara* et al., 1968; *Klupp* et al., 1970). This effect began 15-20 s after the i.v. injection and slightly before changes in heart rate and blood pressure. The recovery paralleled that of the cardiovascular events. Using implanted electrodes, the decrease in splanchnic discharges was also demonstrated in unanesthetized dogs (*Schmitt* et al., 1974). The rate of electric discharge in small fiber bundles of the cervical trunk and of the major splanchnic nerve was quantified by *Klupp* et al. (1970). There was a linear correlation between the logarithm of the dose of clonidine and the percentage inhibition of spontaneous discharge. An inhibition of 50% of the discharge rate was calculated for an i.v. dose of 10.5 μg/kg. The clonidine-induced decrease in sympathetic nerve activity was also observed after section of the main afferent cardiovascular reflex pathways such as the vagus and carotis sinus nerves and the nodose ganglia (*Hukuhara* et al., 1968; *Schmitt* et al., 1968). There was a gradual decrease in splanchnic nerve discharge after the injection of 1-2 μg clonidine/kg into the cisterna magna, the third cerebral ventricle or the lateral cerebral ventricle of cats and dogs (*Schmitt* and *Schmitt,* 1969). The onset of this effect was slower than after i.v. injection: 1-5 min were required for the maximal effect to develop after intracisternal injection. All these experimental results indicate that clonidine suppressed the activity of the sympathetic system by a direct

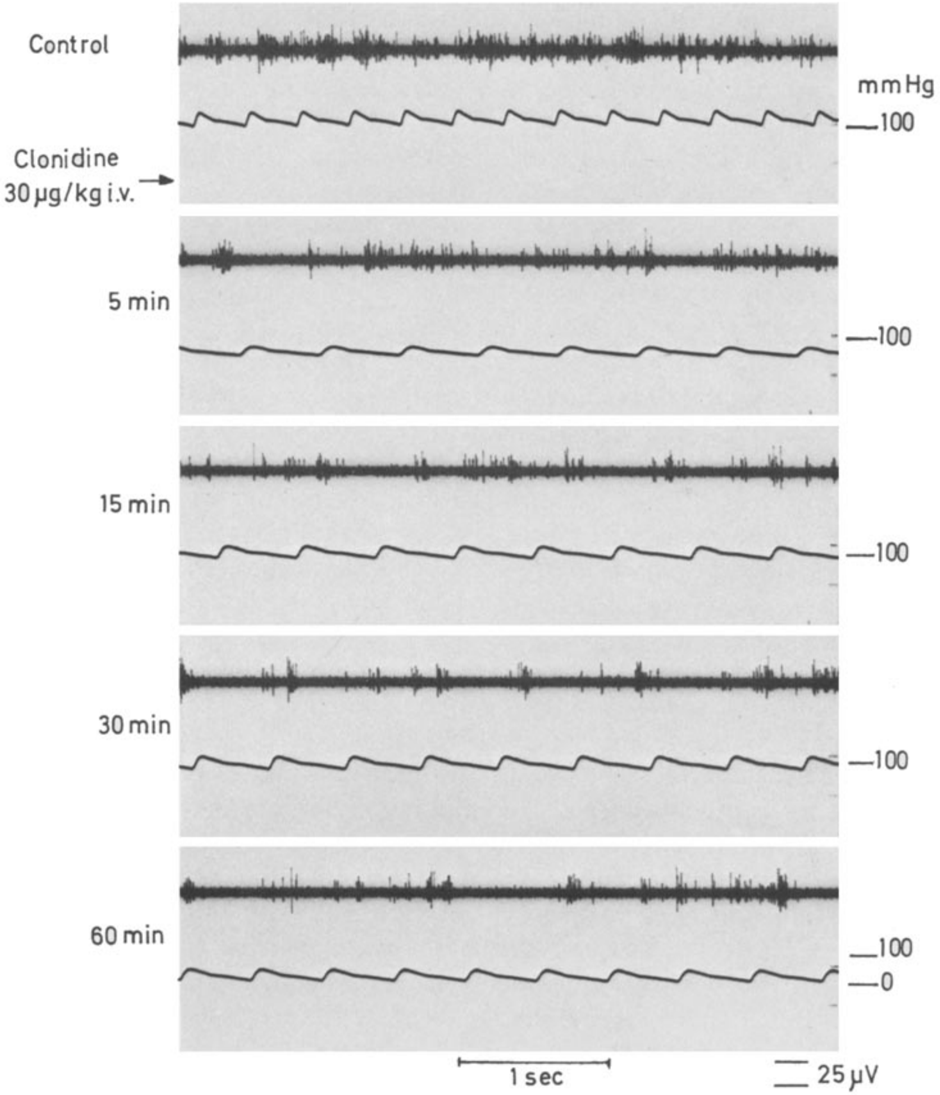


Fig. 6. Effect of clonidine on electric activity in the splanchnic nerve (anesthetized cat). Electric discharges of the left splanchnic nerve (upper trace) and blood pressure (lower curve) before (control), and at different times after, i.v. injection of clonidine. Note decrease in discharge rate, blood pressure and rate of pulse waves

effect upon the CNS. However, the clonidine-induced decrease in spontaneous nerve activity was not uniform in all sympathetic nerve fibers. Discharges of the cervical sympathetic chain were far less attenuated than in splanchnic or cardiac nerves. With some doses, the cardiac nerve activity was less affected than that of the splanchnic nerve (*Schmitt,* 1975). Similar observations were also reported by *Haeusler* (1974a) in cats where 100 µg clonidine /kg virtually abolished the spontaneous activity in the splanchnic and renal sympathetic nerves for several hours, but it only diminished that in the cervical sympathetic trunk for 20-30 min. This differential effect may be explained by the fewer number of vasomotor fibers within the sympathetic cervical trunk as compared with the other sympathetic nerves, and, by the assumption that clonidine preferentially reduces the adrenergic outflow to organs involved in cardiovascular regulation (*Haeusler,* 1974a).

Clonidine also reduced the increase in sympathetic nerve activity induced by stimulation of hypothalamic, medullary or spinal areas as well as the potentials evoked by stimulation of afferent nerves (*Hukuhara* et al., 1968; *Schmitt* et al., 1968; *Klupp* et al., 1970). However, the drug seemed less potent in reducing the induced rather than spontaneous discharges. *Klupp* et al. (1970) determined the effect of hypothalamic electric stimulation (trains of stimuli, 15 sec, 50 Hz) on the activity in fibers of the splanchnic nerve and cervical trunk of cats. The dose of clonidine necessary to reduce this effect by 50% was 25 μg/kg, i.e., significantly higher than the 10.5 μg/kg required for 50% inhibition of spontaneous discharges (see above). Electric stimulation of the posterior hypothalamus increased electric activity in various sympathetic nerves. These responses were markedly reduced by clonidine (100 μg/kg, i.v.) in splanchnic and renal nerves, but not in the cervical sympathetic trunk (*Haeusler,* 1974a). After clonidine, the respiratory rhythm in sympathetic nerve discharges was increased, revealing the greater "resistance" of the central sympathetic neurones to the driving influence of the respiratory center than to the continuous "spontaneous" activity (*Hukuhara* et al., 1968; *Schmitt* et al., 1968). Similarly, the activation of sympathetic nerves by chemoreceptor stimulation (e.g., by the i.v. injection of 0.1 mg/kg nicotine) was not altered by 30 μg clonidine /kg in dogs (*Schmitt* et al., 1968). The spontaneously occurring discharges in renal nerves of cats with respect to two periodic components was investigated by *McCall* and *Gebber* (1976). For 3 and 10 Hz, clonidine decreased both discharges but more in the latter case than in the former. A similar change followed activation of the baroreceptor reflex. These reports show that clonidine does not produce an overall inhibition of the sympathetic nervous system, but that differential effects can be achieved: any increase in sympathetic nerve activity, induced by various stimuli, is less affected than spontaneous nerve activity; vasomotor fibers seem more sensitive to clonidine than those mediating other sympathetic activities; different periodic components within a nerve can be affected differentially.

Striking decreases in the electric discharge rate of sympathetic nerves were reported for xylazine (0.25-1 mg/kg, i.v.) in various species. Xylazine, like clonidine, exerted differential effects with respect to different nerves and to spontaneous and evoked sympathetic nerve activity (*Schmitt* et al., 1970).

Guanabenz reduced spontaneous firing rate in the renal sympathetic nerve of debuffered cats, but very high doses were required to reduce the nerve response to stimulation of the posterior hypothalamus (*Baum* and *Shropshire,* 1976). Similarly, BS 100-141 decreased the spontaneous discharge of splanchnic nerve fibers in cats. The cumulative i.v. dose producing a 50% reduction of nerve activity was 83 μg/kg as compared with 3.4 μg/kg for clonidine (*Waite,* 1975).

2.4.2.3. Inhibition of Cardiovascular "Pressor" Reflexes. A number of reflexes (which are mainly an expression of an activation of the sympathetic nervous system) are decreased by clonidine-like drugs but the responses are not uniform. Clonidine depressed the reflex increase in blood pressure following occlusion of the common carotid arteries in anesthetized dogs (*Hoefke* and *Kobinger,* 1966, see Figure 3; *Boissier* et al., 1968; *Schmitt* et al., 1968), was less effective in cats (*Sattler* and *van Zwieten,* 1967; *Bentley* and *Li,* 1968; *Rand* and *Wilson,* 1968; *Li* and *Bentley,* 1970) and enhanced the reflex in rats (*Bentley* and *Li,* 1968). Orthostatic hypotension, induced by tilting of anesthetized animals, was not enhanced by clonidine in doses which decreased resting blood pressure and heart rate (*Constantine* and *McShane,* 1968; *Nolan* and *Bentley,* 1975). Higher doses of clonidine (>8 μg/kg) accentuated hypotensive responses during the tilting manoeuvre (*Constantine* and *McShane,* 1968; *Maling* et al., 1969). A Valsalva-like manoeuvre in conscious rabbits caused a reflex rise in total peripheral resistance (*Korner* et al., 1976). The response was attenuated by clonidine, in a dose-dependent manner, when the drug was given either i.v. or into the lateral ventricle. In isolated perfused hindquarters of cats, a reflex vasoconstriction was elicited by injection of vasodilating drugs into the upper part of the body. This pressor reflex was abolished by prior treatment with guanethidine or reserpine; however, it was augmented by clonidine in spite of the hypotensive effect of the drug (*Li* and *Bentley,* 1969; 1970). In conscious ducks, the diving reflex is produced by submerging the beak of the animal in water. The cardiovascular response consists of maximum bradycardia and reduction of cardiac output but the mean blood pressure remains unchanged due to an increase in sympathetic vasoconstriction (*Folkow* et al., 1967). After treatment with guanethidine, bretylium or reserpine, the blood pressure fell to low levels during "diving." Although clonidine (100-200 μg/kg) or α-methyldopa also decreased the resting blood pressure, the mean blood pressure remained constant during the experimental dive (*Kobinger* and *Oda,* 1969).

A difference between pressor responses to weak and strong reflex stimuli has been demonstrated in unanesthetized, midbrain-sectioned rabbits (pontine preparation; *Shaw* et al., 1971). The rise in blood pressure and total vascular resistance in response to lowering the arterial oxygen tension to 50 mm Hg (mild hypoxia) was abolished by clonidine (20 μg/kg + infusion of 1.5 μg/kg/min). However, the response to severe hypoxia (oxygen tension 30 mm Hg) was only partially decreased by the drug.

Schmitt et al. (1970) and *Antonaccio* et al. (1973) reported that xylazine reduced the effect of carotid artery occlusion on blood pressure and heart rate of dogs but did not attenuate the increase in splanchnic nerve discharge.

In humans, clonidine-induced decreases in blood pressure were the same in the supine and erect position. Severe orthostatic side effects did not occur (*Grabner* et al., 1966; *Onesti* et al., 1971; *Bock* et al., 1973; *Schwartz* et al., 1973). Clonidine had little effect on the blood pressure response to a Valsalva manoeuver but caused a slight decrease in the pressor response to immersion of one hand into ice-cold water (*Dollery* et al., 1976). It may be concluded, therefore, that clonidine-like centrally acting drugs decrease the resting tone of the sympathetic system but still permit the passage of vital reflex adjustments. Apparently, these drugs do not block the final efferent sympathetic vasomotor neurones of the medulla (*Kobinger* and *Oda*, 1969, *Klupp* et al., 1970) but exert a modulatory effect upon them. In this respect, the inhibitory effect of centrally acting drugs is different from that of substances which block the adrenergic neurones at peripheral sites: the latter interfere severely with "pressor reflexes" and may cause considerable orthostatic hypotension in humans.

2.4.2.4. Facilitation of Cardiovascular "Inhibitory" Reflexes. *Robson* and *Kaplan* (1969) and *Robson* et al. (1969) induced reflex bradycardia in dogs by the i.v. injection of pressure-raising catecholamines, and showed that clonidine (20 μg/kg, i.v.) facilitated the reflex. These results were obtained in animals in which the sympathetic innervation of the heart had been blocked by ß-adrenoceptor-blocking agents or by guanethidine. Thus, the results indicated an activation of the vagally mediated cardiodepressor reflex. Similar results were obtained by *Nayler* and *Stone* (1970). This baroreceptor stimulation might be due to the peripheral vasoconstricting effect of the drug (*Heymans* and *Neil*, 1958; see Section 2.4.1.). However, it was shown to be a central action. Clonidine (1 μg/kg) was injected either intracisternally or i.v. into dogs treated with a β-adrenoceptor antagonist. The bradycardia elicited by the i.v. injection of angiotensin was enhanced after intracisternal injection, but clonidine given i.v. was ineffective (Figs. 7 and 8; *Kobinger* and *Walland*, 1971; 1972a). Intracisternally applied clonidine caused little or no decrease in the resting heart rate under these experimental conditions. Analogous results were obtained in conscious dogs and in dogs in which the baroreceptors in the upper part of the body were stimulated by a blood pressure rise (induced by an inflatable rubber cuff around the descending aorta; *Walland* et al., 1974).

All these results clearly showed that clonidine activated central components of the baroreceptor reflex arc.

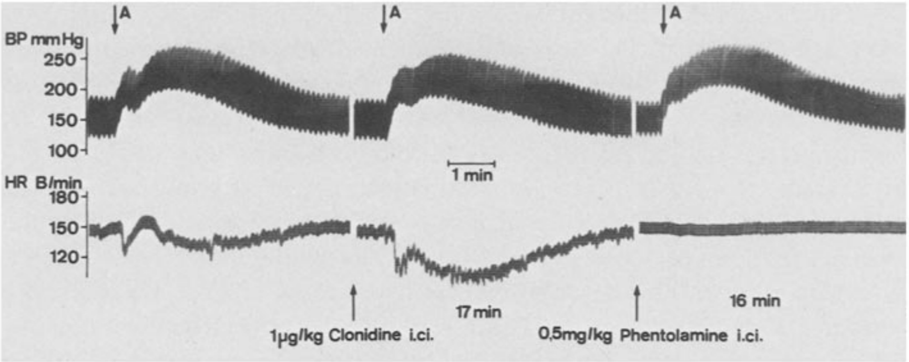


Fig. 7. Facilitation of the baroreceptor reflex by clonidine. (Anesthetized dog given gallamine and artificial respiration) β-adrenoceptors were blocked (toliprolol) to exclude adrenergic responses of the heart. Intravenous injection of angiotensin (A, 0.1 µg/kg) increases the blood pressure (BP) and this reflexely decreases the heart rate (HR, B=beats). Intracisternal (i.ci.) injection of clonidine facilitates this reflex and the α-adrenoceptor antagonist phentolamine (40 min later) abolishes the effect. The times refer to the preceding i.ci. injection. From *Kobinger* (1974)

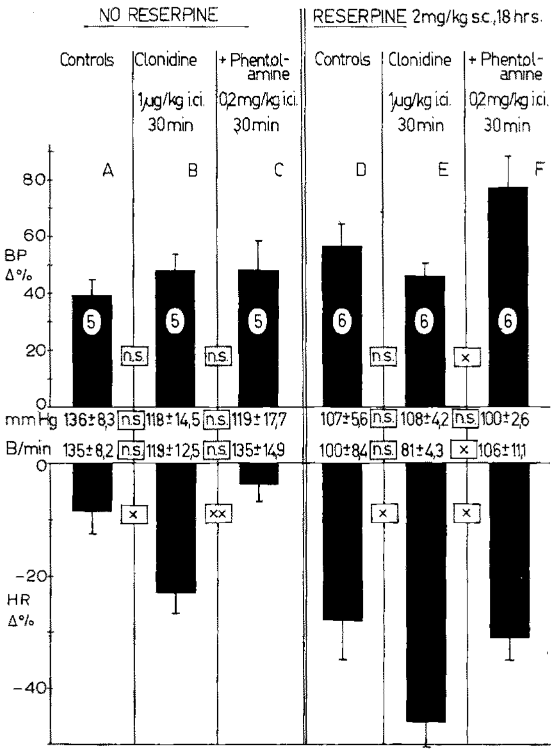


Fig. 8. Facilitation of the baroreceptor reflex by clonidine. Anesthetized dog with spontaneous respiration and β-adrenoceptor blockade with toliprolol). Mean blood pressure and mean heart rate (B = beats) are given between the columns (± S.E.M.). The baroreceptor reflex was elicited by i.v. injections of angiotensin (0.025-0.3 µg/kg). The resulting maximal changes are expressed as the % of the values before angiotensin and are given by the columns as mean ± S.E.M. BP = blood pressure, HR = heart rate.

In B, C, E and F, angiotensin was injected 30 min after intracisternal injection of the drugs. Numbers of dogs are indicated within columns. The significance of differences is indicated between those groups which have been compared: xx, p < 0.01; x, P < 0.05; n.s., not significant (p > 0.05). In D, E and F, dogs were pretreated with reserpine (2 mg/kg, s.c.) 18 h before the experiment. From *Kobinger* and *Walland* (1973)

Clonidine facilitates the baroreceptor reflex in the vagus and also in the sympathetic system. The drug enhanced the decrease in electric discharge in sympathetic nerves after electric stimulation of the afferent carotid sinus nerve (*Haeusler,* 1974a). An extensive study of the relation between baroreceptor activation and heart-rate response before and after clonidine was carried out by *Korner* et al. (1974) in conscious rabbits. By means of inflatable balloons around the abdominal aorta and the inferior caval vein, graded changes in blood pressure were produced. The results indicated that the drug acted mainly on those efferent cardiac neurones which receive afferent projections from baroreceptors. There was only a small influence on neurones which do not receive baroreceptor projections. Comparison of results in vagotomized and normal rabbits revealed that clonidine affected the baroreceptor-sensitive neurones by enhancing both vagal excitation and sympathetic inhibition, while the baroreflex-independent effect was entirely due to cardiac sympathetic inhibition. In dogs, the cardiodepressor reflex facilitation by clonidine apparently involves mainly projections from the aortic baroreceptors since the drug was ineffective 20 days after aortic nerve denervation (*Antonaccio* et al., 1975).

An enhancement of reflex bradycardia after i.v. injection has been reported for xylazine (*Antonaccio* et al., 1973; dog) and for St 93, tolonidine and flutonidine (*Hoefke* et al., 1975; rat)

2.4.2.5. Response Pattern of the Autonomic Nervous System. From the previous sections, it appears that clonidine-like drugs simultaneously increase vagal and decrease sympathetic activity by a direct action on the CNS (Fig. 9).

A reciprocal reaction of both parts of the autonomic nervous system is integrated into the complex response patterns which are triggered by physiologic demands. Such coordinated responses follow stimulation of hypothalamic and cortical centres. A decrease in sympathetic and an increase in vagal activity is observed during stimulation of the "sympathoinhibitory" areas of the anterior hypothalamus, of the "depressor" areas of the anterior cingulate gyrus, of certain parts of the medulla ("depressor area of the vasomotor centre") and of afferent baroreceptor pathways (for reviews see *Uvnäs,* 1960; *Löfwing,* 1961; *Folkow* and *Neil,* 1971). The hypotensive effect of clonidine was thought to be due to activation of the central pathway of the baroreceptor reflex (*Schmitt,* 1971; *Haeusler,* 1973). However, this may be only one of the possible sites of action of this type of drug. The activation of suprabulbar projections (even at the bulbar level) must be taken into account. A more detailed analysis of the activation of the central arc of the baroreceptor reflex by clonidine and other α-adrenoceptor stimulating drugs is given in Section 5 (see also Fig 13).

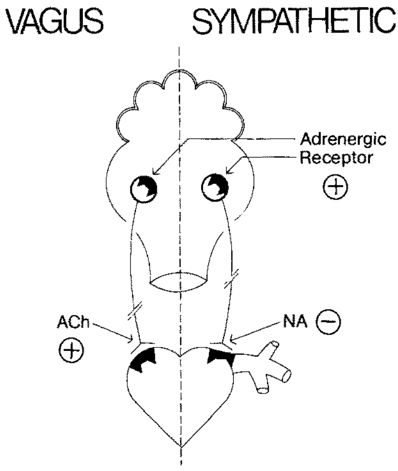


Fig. 9. Schematic representation of the medullary autonomic nervous system and the cardiovascular system. The symbols ▼ denote receptors. ACh = acetylcholine, liberated at vagal nerve endings in the heart. NA = noradrenaline, liberated at sympatho-adrenergic nerve endings in the heart and at vascular sites. Activation of central adrenoceptors (⊕) decreases the activity at peripheral adrenoceptors (⊖), and increases the activity at peripheral vagal cholinergic nerve endings (⊕). From *Kobinger* (1974)

2.4.2.6. Localization of the Central Site of Cardiovascular Depression. A great number of experiments have shown that clonidine acts at various levels of the CNS. The most prominent site seems to be the oblongate medulla since low doses of clonidine decreased blood pressure, heart rate and sympathetic nerve activity after transection of cat brain at various levels between pons and the rostral level of the medulla (*Hukuhara* et al, 1968; *Schmitt* and *Schmitt,* 1969; *Klupp* et al., 1970). The facilitation of the vagally mediated baroreceptor reflex of dogs and rats was not diminished by midbrain transection or by additional removal of the cerebellum (*Kobinger* and *Pichler,* 1975a,b).

An effect of clonidine on preganglionic sympathetic neurones was demonstrated at the spinal medullary level since spontaneous activity in sympathetic nerves was diminished in cats with high spinal transection (*Hukuhara* et al., 1968; *Sinha* et al., 1973). Higher doses were necessary to decrease the activity at spinal than at medullary sites. Moreover, clonidine decreased the sympathetic nerve activity evoked by stimulation of afferent spinal nerves or of the (descending) dorsolateral column in spinal cats (*Sinha* et al., 1973; *Franz* et al., 1975; *Haeusler,* 1976). Again, higher doses of clonidine were necessary to affect the spinal pathways than to affect those which pass the medulla. The splanchnic nerve potentials, evoked by electric stimulation of the cervical cord in spinal cats, were diminished by 30 μg clonidine/kg whereas, in intact cats, the late potential evoked by afferent stimulation of the sciatic nerve (which is known to use a spino-medullary-spinal pathway) was reduced or abolished by 3 μg clonidine/kg (*Sinha* et al., 1973). A diencephalic site of action of clonidine, in addition to the medullary site, was proposed by *Shaw* et al. (1971) because the cardiovascular effects of the drug were not identical in control rabbits and in those with mesencephalic transection. An action of the drug in regions of the forebrain was also postulated on the basis of the following experiments in vagotomized cats: electric stimulation of the medullary reticular formation increased blood pressure and this was reduced by clonidine. Subsequent midbrain transection reestablished the stimulation-induced pressor response (*Klevans* et al., 1973). Structures rostral of the medulla and of the hypothalamus were hypothesized to be sites of a pressor action of clonidine. After midbrain or prehypothalamic transections in unanesthetized rats, the decrease in blood pressure was more pronounced than in intact animals (*Trolin,* 1975; *Henning* et al., 1976). It must be noted that central transections may result in the "resetting" of medullary activity by elimination of (sympatho-) inhibitory pathways (see *Korner,* 1971). These changed conditions make it very difficult to draw any conclusions concerning the action which drugs may exert rostrally of a transection level.

A more precise localization has been tried by the local administration or injection of drugs. A medullary site of action was indicated by the fall in blood pressure when clonidine was applied topically to the floor of the fourth ventricle of cats by means of tissues soaked with 0.01% - 1% solutions of the drug (*Schmitt* and *Schmitt,* 1969; *Dhawan* et al., 1975). A blood pressure fall was also reported after the application at "chemosensitive zones" on the ventral surface of the brain stem (*Bousquet* and *Guetzenstein,* 1973). Guanabenz had no effect when applied to this region, so a different site of action from clonidine was postulated for this drug (*Scholtysik* et al., 1975). A technique was developed by *Philippu* et al. (1973) whereby circumscribed areas of cat brain could be superfused with drug solutions using a double-barreled cannula ("push-pull" cannula). Electric stimulation of the posterior area of the hypothalamus increased the blood pressure and this was dose-dependently inhibited by superfusion of the area of the ipsilateral nucleus of the solitary tract with clonidine (10^{-3}-10^{-1} *M*). Stereotactic injections of clonidine (>3 μg) into rat hypothalamus induced hypotension and bradycardia indicating an effect on forebrain structures (*Struyker Boudier* and *van Rossum,* 1972). These effects were produced over a large area of the hypothalamus, whereas for noradrenaline the action was localized to the anterior hypothalamic/preoptic region (*Struyker* et al., 1964a). Pressor responses to electric stimuli were evoked from the tip of the push-pull cannula located in the posterior hypothalamus of cats (*Philippu* et al., 1974). This effect was enhanced by superfusion with low concentrations of clonidine (10^{-5}-5 x 10^{-5} *M*) but was reduced by high concentrations (10^{-3}-10^{-2} *M*).

It should be noted that the interpretation of results obtained with local administration or injection of clonidine-like drugs is limited by their local anesthetic action (see Section 2.2.5.). For example, procaine causes a fall in blood pressure when applied to the ventral surface of the medulla (*Loeschke* and *Koepchen,* 1958).

Electrophysiologic data suggest that the nucleus tractus solitari is a first "relay station" of the baroreceptor pathway (*Cottle,* 1964; *Crill* and *Reis,* 1968; *Seller* and *Illert,* 1969). This nucleus has been suggested as a site of action of clodine, because the drug activates central components of the baroreflex arc (*Schmitt* et al., 1971; *Kobinger* and *Walland,* 1973). Destruction of a depressor area at the floor of the fourth ventricle (just rostral to the obex in the midline) attenuated the depressor effect of clonidine in dogs. This indicates a site of action in the area of the nucleus tractus solitarii but does not exclude other possibilities (*Schmitt* et al., 1973a).

The clonidine-like hypotensive drugs, flutonidine and tramazoline, also decreased blood pressure and heart rate after injection into the anterior hypothalamic region of rat brain (*Struyker Boudier* et al., 1974b; 1975a).

2.4.2.7. Hypotension in Humans. The question arises whether the central mechanisms demonstrated in animals are responsible for the antihypertensive effect in man during chronic treatment with clonidine (*Zaimis* and *Hanington,* 1969; *Katic* et al., 1972). Under a therapeutic regimen, a reduction of the sympathetic activity was clearly shown by *Hökfelt* et al. (1970; 1975). These authors treated hypertensive patients with clonidine and reported a reduction in urinary and plasma catecholamines that was closely related to the fall in blood pressure and heart rate. The absence of severe orthostatic side effects distinguished the drug from peripheral adrenergic blocking agents (for references see Section 2.4.2.3.). A central action of clonidine in man, depending on the integrity of descending bulbospinal pathways, was recently demonstrated in tetraplegic patients with complete lesions above the level of the sympathetic outflow (*Reid* et al., 1976). Following oral ingestion of 0.3 mg clonidine there was no significant fall in blood pressure but the bradycardia was more pronounced (vagus intact), and the sedation and reduction in saliva production was about normal.

3. α-Methyldopa

A survey of older reviews on α-methyldopa (DL-α-methyl-3,4-dihydroxyphenylalanine) reveals that the biochemical effects of this drug attracted researchers much earlier than the pharmacologic effects (see *Holtz* and *Palm,* 1966; *Muscholl,* 1966). Thus, as the drug decreased the noradrenaline content of heart and brain in laboratory animals (*Porter* et al., 1961), the hypotensive effect of α-methyldopa (first reported in man by *Oates* et al., 1960) was explained by a lack of noradrenaline. There follows a review of the hypothesis that α-methyldopa lowers blood pressure through its metabolite α-methylnoradrenaline which stimulates α-adrenoceptors in the CNS. This hypothesis of the mode of action of α-methyldopa was recently called the "pressor approach to depressor therapy" (*Sjoerdsma,* 1975), and was developed on the basis of an increasing knowledge of the mode of action of clonidine. The subject has been reviewed previously by *Henning* (1969a) and by *van Zwieten* (1975a).

All hypotheses concerning the mode of action of α-methyldopa require knowledge of the metabolic changes of the drug as depicted in Figure 10. The figure also shows the last steps of the synthesis of the natural transmitter, noradrenaline. Hypotensive and biochemical effects of α-methyldopa, such as inhibition of dopa decarboxylase and depletion of tissue noradrenaline, are entirely due to the L isomer (*Porter* et al., 1961; *Gillespie* et al., 1962).

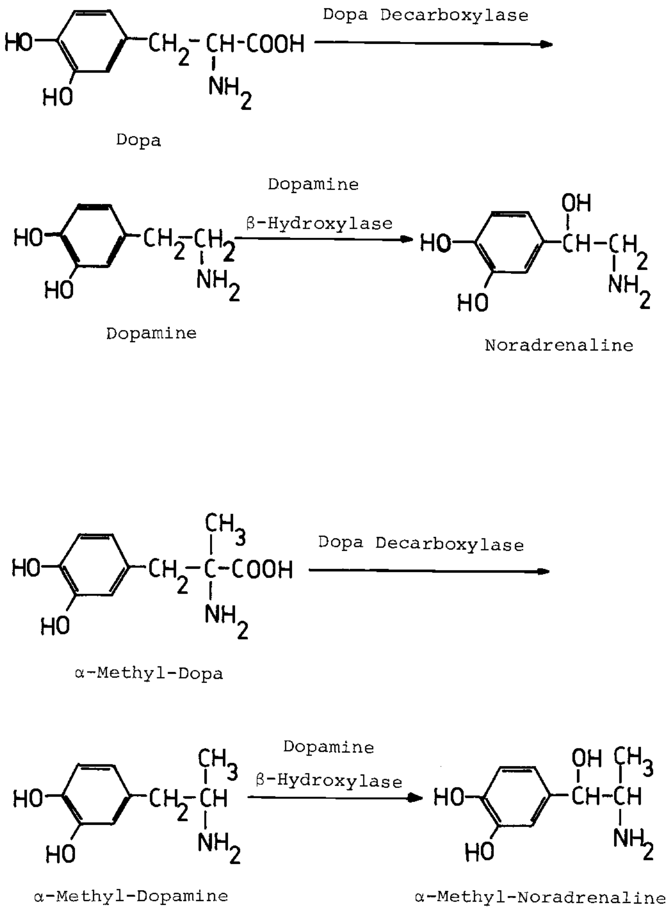


Fig. 10. Enzymatic conversion of dopa into the transmitter, noradrenaline, and of the hypotensive drug α-methyldopa into the active compound, α-methylnoradrenaline

3.1. Cardiovascular Reaction Pattern and Peripheral Effects

There are only a few studies of the hemodynamic effects of α-methyldopa in animals, probably because the hypotensive effect is not easily demonstrable in acute experiments in anesthetized normotensive animals (*Kroneberg,* 1962-63; *Tauberger* and *Kuhn,* 1971). Acute hemodynamic studies, using α-methyldopa in hypertensive patients, revealed a blood pressure decrease together with bradycardia and, in most instances, either a decrease in total peripheral vascular resistance or a decrease in cardiac output. Thus, the effects of α-methyldopa resembled those of adrenergic blocking agents (for review see *Kirkendall* and *Wilson,* 1962; *Sannerstedt* and *Conway,* 1970). A causal relationship between lowering of the blood pressure by α-methyldopa and an overall decrease in sympathetic nerve activity, in hypertensive patients, was demonstrated in a study by *Muscholl* and *Rahn* (1968): single and repeated treatment with α-methyldopa caused a decrease in renal excretion of free noradrenaline which correlated with a fall in systolic blood pressure.

Hypotension induced by α-methyldopa was originally believed to be due to inhibition of dopa decarboxylase. However, this hypothesis was proven wrong (*Porter* et al., 1961). A second proposal was that the conversion of α-methyldopa to the false transmitter, α-methylnoradrenaline, interfered with peripheral sympathetic transmission. This hypothesis was mainly supported by biochemical findings showing stoichiometric displacement of noradrenaline by α-methylnoradrenaline and release of the latter by electrical stimulation of sympathetic nerves (*Carlsson* and *Lindquist,* 1962; *Muscholl* and *Maître,* 1963). This hypothesis turned out to be untenable because, under various experimental conditions of electric or reflex stimulation of sympathetic nerves, α-methyldopa caused little or no diminution of the responses (*Goldberg* et al., 1960; *Day* and *Rand,* 1964; *Kobinger* and *Oda,* 1969). However, the presence, at peripheral sites, of the false transmitter having 0.1 - 1 times the potency of the natural transmitter (*Muscholl* and *Maître,* 1963; *Day* and *Rand,* 1964; *Pettinger* et al., 1963), might be a minor factor contributing to the antihypertensive effect of α-methyldopa.

3.2. Central Site of Action

A central site of antihypertensive action was proposed in connection with the sedative side effects of α-methyldopa (*Oates* et al., 1960; *Gillespie* et al., 1962).

A more direct analysis of the central site of action was carried out by *Jaju* et al. (1966) and *Henning* and *van Zwieten* (1967; 1968). The latter authors infused 20 mg L-α-methyldopa /kg body wt. into the vertebral artery of anesthetized cats during one hour. Hypotension ensued which, though slow in onset, lasted for at least 4-5 h. Control experiments showed that the i.v. infusion of 20 mg L-α-methyldopa/kg body wt. to be only slightly hypotensive and that the intraarterial infusion of 20 mg/kg of the D-isomer was ineffective. Amine concentrations were determined 3 h after the end of the infusion. There was no significant change in the noradrenaline content of the heart. However, the noradrenaline and dopamine content of the brain was decreased by about the same degree after i.a. and after i.v. treatment with L-α-methyldopa, an observation which was surprising in view of the different effects on the blood pressure (*Henning* and *van Zwieten,* 1968). A number of papers confirmed the hypotensive effect of centrally infused or injected α-methyldopa (*Ingenito* et al., 1970; *Day* et al., 1973; *Finch* and *Haeusler,* 1973). The central sympathoinhibitory effect of α-methyldopa was also indicated by the decrease in spontaneous sympathetic nerve discharge (*Tauberger* and *Kuhn,* 1971; *Baum* et al., 1972). When the drug was administered repeatedly for several days to cats and rats, renal hypertensive animals responded to lower doses than normotensive animals did (*Baum* et al., 1972). There was, however, no decrease in blood pressure and sympathetic outflow after a single dose of α-methyldopa (100-400 mg/kg, i.v.) in normotensive rats and cats (*Tauberger* and *Kuhn,* 1971).

The next observation was that the decarboxylation of α-methyldopa to the corresponding amines within the central nervous system is a prerequisite for its hypotensive action (*Henning,* 1969b). Rats were treated with one of two inhibitors of dopa decarboxylase: seryl-2, 3, 4-trihydroxybenzylhydrazine (Ro 4-4602; *Pletscher* and *Gey,* 1963) which inhibits both the peripheral and central nervous decarboxylase, or, α-hydrazino-α-methyl-β-(3, 4-dihydroxyphenyl)-propionic acid (MK 485) which does not readily penetrate the blood brain barrier (*Porter* et al., 1962). It was shown that in conscious renal hypertensive rats, the hypotensive effect of α-methyldopa was prevented by treatment with Ro 4-4602 but not changed by treatment with MK 485. Both inhibitors prevented the formation of α-methyldopamine in rat heart, while in brain the formation was significantly reduced by Ro 4-4602 only. The content of α-methylnoradrenaline was not determined in these studies, but it was supposed the content was influenced in the same way as that of its precursor, α-methyldopamine. This supposition was proved correct by pretreatment of animals with inhibitors of dopamine β-hydroxylase such as bis-(4-methyl-1-homopiperazinyl-thiocarbonyl) disulfide (FLA-63), disulfiram, sodium diethyldithiocarbamate or 1-phenyl-3-(2-thiazolyl)-2-thiourea (U-14, 624). All these prevented the hypotensive effect of α-methyldopa in normotensive and hypertensive rats. Thus, the formation of α-methylnoradrenaline is necessary to mediate the hypotensive effect of α-methyldopa (*Henning* and *Rubenson,* 1971; *Day* et al., 1973). The essential site of metabolism is within the CNS since the experiments were carried out after inhibition of the peripheral decarboxylase (*Henning* and *Rubenson,* 1971). A dopamine β-hydroxylase inhibitor (U-14, 624) which acts preferentially in the CNS, abolished the fall in blood pressure in response to α-methyldopa (*Day* et al., 1973). The importance of the integrity of cerebral adrenergic neurones for the conversion of α-methyldopa to α-methylnoradrenaline was indicated by the results of *Finch* and *Haeusler* (1973): prior intracerebroventricular administration of 6-hydroxydopamine abolished the hypotensive response to α-methyldopa in conscious genetically hypertensive rats. As clonidine was still effective after the same pretreatment (*Haeusler* and *Finch,* 1972; *Finch,* 1975), the effect of 6-hydroxydopamine must be specific (see Section 4.4.).

One has to consider whether the central effect of α-methyldopa is due to the displacement and therefore to a lack of central noradrenaline, or due to the presence and action of the α-methylated amines within the CNS. The following observations are against the displacement hypothesis: the amino acid, α-methyl-m-tyrosine, readily penetrates the blood brain barrier and depletes noradrenaline in brain and peripheral tissues even more actively than does α-methyldopa (*Porter* et al., 1961; *Henning,* 1969a). α-Methyl-m-tyrosine is metabolized to α-methyl-m-tyramine and to metaraminol. The latter displaces endogenous noradrenaline and is liberated instead of the natural transmitter (*Carlsson* and *Lindquist,* 1962). The pressor activity of metaraminol in rats is about 1/6 that of α-methylnoradrenaline and 1/20 that of noradrenaline (*Brunner* et al., 1967). If the "false transmitter" theory holds true for the CNS, α-methyl-m-tyrosine should be more effective than α-methyldopa as a hypotensive agent, but it proved ineffective in this respect (*Henning,* 1967). Further, there is no correlation between the time course of the effect of α-methyldopa on blood pressure and the effect on the depletion of noradrenaline in the brain after infusion of the drug into the vertebral artery of cats (*Henning* and *van Zwieten,* 1968). A "direct" effect of α-methylnoradrenaline was indicated by experiments in which tissue noradrenaline was depleted by pretreatment with α-methyl-m-tyrosine and α-methyl-p-tyrosine (an inhibitor of tyrosine hydroxylase). The procedure did not diminish the hypotensive effect of α-methyldopa (*Henning* and *Rubenson,* 1971).

The suggestion that stimulation of central adrenoceptors, by one of the sympathomimetic metabolites of α-methyldopa, mediates the hypotension was made by *Hoyer* and *van Zwieten* (1971; 1972) and by *Henning* and *Rubenson* (1971) on the basis of results obtained with other adrenergic stimulating substances (clonidine, amphetamine, α-methyldopa and others). An important contribution was the proof that α-methylnoradrenaline, when administered directly into the CNS, decreased the blood pressure and that this effect was antagonized by α-adrenoceptor blocking agents (*Heise* and *Kroneberg,* 1972; *de Jong* et al., 1975). The validity of this hypothesis depends on the effectiveness of α-methylnoradrenaline as a stimulator of α-adrenoceptors in peripheral tissues (see above) and in the CNS; this will be reviewed in the following sections.

4. Central α-Adrenoceptors as Mediators of Cardiovascular Depression

The strong and specific stimulation of peripheral α-adrenoceptors by clonidine and related drugs has been a challenge for pharmacologists trying to explain, by the same receptor mechanism, the hypotensive effects within the CNS (*Schmitt* et al., 1968; *Heise* et al., 1971). The idea was supported by *Andén* et al. (1970) who reported that clonidine exerted some noncardiovascular CNS effects by an action on central α-adrenoceptors; i.e., the drug facilitated the somatic flexor reflex in hindlimbs of spinal rats, an action which was previously shown to be due to a specific stimulation of this receptor type within the spinal medulla.

The conclusion that the cardiovascular inhibitory effects of centrally acting hypotensive drugs of the clonidine type are mediated by central α-adrenoceptors rests on two types of experiments in which: (1) typical effects are antagonized by α-adrenergic blocking agents, (2) typical effects are mimicked by a variety of α-adrenergic stimulants (including noradrenaline) administered directly into the CNS.

4.1. Effect of α-Adrenoceptor Antagonists

The only experiments that can prove the above hypothesis are those in which the antagonists do not interfere with peripheral adrenergic pathways secondarily involved in the effect of the hypotensive agents. A diminished hypotensive effect of clonidine-like drugs, as a result of systemic pretreatment with α-adrenoceptor blocking substances, is not a valid argument for a central antagonism and might be better explained by diminished sympathetic effect at peripheral vascular sites (*Kobinger* and *Walland,* 1967a; *Heise* et al., 1971). The hypothesis was proved by the following experiments in which interference of the α-adrenoceptor blockers with peripheral receptors did not affect the aim of the experiment. *Schmitt* et al. (1971, 1973b) reported that the electric discharge of the splanchnic nerve was not consistently changed by piperoxan or yohimbine. This treatment, however, prevented the decrease in nerve activity usually caused by clonidine (see Section 2.4.2.2.). When clonidine was injected first, the α-adrenoceptor blocking agents were able to reestablish electric nerve activity. The nerve activity was paralleled by changes in blood pressure and heart rate, and the antagonism was shown after i.v. or after cerebral administration (intracisternal, lateral ventricle, vertebral artery) in cats as well as in dogs. Repeated injections of various doses of agonist and antagonists indicated that the antagonism was of the competitive type. Another approach was to determine the facilitatory effect of intracisternally injected clonidine on the vagally mediated baroreceptor reflex (see Section 2.4.2.4.). When the reflex had been enhanced by intracisternally injected clonidine, it was antagonized by the subsequent i.v. or intracisternal injection of one of several antagonists: phentolamine, chlorpromazine or haloperidol (Figs. 7 and 8; *Kobinger* and *Walland*, 1971; 1972b). The same dose of phentolamine which was effective intracisternally (0.5 mg/kg) was ineffective intravenously, proving the central site of the antagonism. The site was later localized in the medulla since piperoxan as well as phentolamine antagonized the vagal baroreceptor effect of clonidine in midbrain-transected dogs and rats (*Kobinger* and *Pichler*, 1975a, b). In these studies, as well as in those of *Schmitt* and colleagues (see above), no α-adrenoceptors are interposed between the CNS and the effector system under experimental observation. Treatment of cats with haloperidol (1.0 mg/kg, i.v.) diminished the hypotensive and bradycardic effect of clonidine but did not decrease its (peripherally induced) initial pressor effect. Similar results were obtained by treatment with phenoxybenzamine (5-10 mg/kg) but not with pimozide and spiroperidol, and were explained by an antagonism at central noradrenaline receptors (*Bolme* and *Fuxe*, 1971). Analogous results were reported later with the clonidine-like drug, guanabenz (*Bolme* et al., 1973). The results of *Schmitt* et al. (1971) were confirmed by *Haeusler* (1973) who recorded the splanchnic and renal nerve discharge in cats and demonstrated that piperoxan antagonized the effect of clonidine. In conscious renal hypertensive cats, the intraventricular injection of clonidine or xylazine (18-112 nmol or 0.07 - 0.14 μmol, respectively) decreased blood pressure and heart rate. Prior intraventricular treatment with phentolamine (0.3-6 μmol) or other α-adrenoceptor antagonists (*Finch*, 1974; 1975) antagonized the effect. As there were no controls to exclude the possibility of a peripheral effect of the antagonists, a central antagonism can be deduced from the heart rate observations but not from blood pressure measurements. A similar objection must be made to experiments in which the fourth ventricle of cats was perfused with phentolamine or phenoxybenzamine followed by xylazine (*Heise* et al., 1971). The percentage decrease in blood pressure produced by xylazine was diminished by the treatment. However, no data indicates or excludes peripheral α-adrenoceptor blockade due to possible "leakage" of the antagonists to peripheral vascular sites.

An antagonism between clonidine and α-adrenoceptor antagonists, with respect to cardiovascular parameters, was also reported at the hypothalamic level: stereotactic injections of phentolamine (106 nmol in 3 μl) into the anterior hypothalamic preoptic region reduced the bradycardia (and hypotension) induced by clonidine (15 or 40 nmol) injected 20 min later at the same site (*Struyker Boudier* et al., 1974a).

In spinal cats, the inhibitory effect of clonidine (and L-dopa) on spontaneous or electrically evoked sympathetic nerve discharges was antagonized by piperoxan (1 μg/kg, i.v.) or yohimbine (0.25 μg/kg, i.v.); this was interpreted as an effect on the spinal α-adrenoceptors which inhibit medullary sympathetic neurones (*Sinha* et al., 1973). Similarly, *Franz* et al. (1975) showed that the clonidine-induced inhibition of sympathetic nerve discharge in spinal cats was antagonized by tolazoline (5 mg/kg, i.v.). Surprisingly, this was not interpreted as an antagonism at noradrenaline receptors, but rather at spinal 5-HT receptors, since the authors found inhibitory 5-HT receptors at sympathetic preganglionic neurones. Statements concerning the specifity of the receptors are limited by the well-known fact that α-adrenoceptor blocking agents can also block 5-HT and/or dopamine receptors. The hypothesis that central α-adrenoceptors mediate the cardiovascular effects of the hypotensive drugs under discussion therefore requires the support by the experiments discussed in the following section.

4.2. Effect of Centrally Applied α-Adrenoceptor Agonists

This section is concerned with substances which exert α-adrenergic effects at peripheral sites and which do not usually decrease blood pressure and heart rate after systemic injection, but rather *increase* one or both parameters. These substances either directly stimulate α- and β-adrenoceptors (adrenaline, noradrenaline, α-methylnoradrenaline) or act "indirectly" by liberation of endogenous noradrenaline (amphetamine, ephedrine, phentermine, chlorphentermine). For definition of the peripheral adrenergic mode of action of these drugs see *Ahlquist* (1948), *Trendelenburg* (1963) and *Holtz* and *Palm* (1966). The imidazolines act directly and rather specifically upon α-adrenoceptors, however, α-adrenoceptor blocking, and local anesthetic effects must be considered (see Section 2.2.3. and 2.2.5.). In many experiments, high doses of these substances had to be used; a "leakage" of only a small part into the peripheral cardiovascular system might exert effects which are opposite to the expected CNS effects and might complicate the interpretation of the cardiovascular responses.

In 1933, *Heller* injected 0.1-0.5 mg adrenaline into the cisterna magna of cats and found a fall in blood pressure in some experiments. Hypotension and bradycardia were reported to follow the injection of noradrenaline (0.22 mg) and 5-HT (5 mg) into the cisterna or lateral ventricle of dogs (*McCubbin* et al., 1960) . As vasodilator drugs had opposite effects, it was concluded that these effects depend on changes in local cerebral blood flow and are thus of a nonspecific nature (*Kaneko* et al., 1960). Conversely, the intraventricular injection of noradrenaline also increased the blood pressure, thus supporting the idea that, depending on the dose, adrenoceptor stimulation in the CNS also mediates cardiovascular activation (*Gagnon* and *Melville,* 1968; *Ito* and *Schanberg,* 1974).

Intracisternal injection of high doses of noradrenaline (10-150 μg) in cats decreased blood pressure, heart rate and the electric discharge of the splanchnic nerve, both spontaneous and evoked by afferent nerve stimulation (*Sinha* and *Schmitt,* 1974). As with clonidine, the late component of evoked potentials was more sensitive to treatment than the early component. The effects of noradrenaline were prevented by prior intracisternal injection of α-adrenoceptor antagonists. The same response of the nerve discharge was reported to follow high i.v. doses of noradrenaline (10-20 μg/kg) injected into animals with the afferent buffer nerves cut to exclude reflex changes in nerve activity. Perfusion of the third and fourth ventricle system of cats with α-methylnoradrenaline, α-methyldopamine or α-methyldopa (30 μg/min, 10 min) decreased the blood pressure. The decrease was practically abolished by the additional infusion of yohimbine or phentolamine (42 and 30 μg/min, respectively; *Heise* and *Kroneberg,* 1972). Similar results were obtained by *Finch* and *Haeusler* (1973) and *Finch* et al. (1975) in hypertensive rats and cats. The blood pressure was lowered by α-methyldopa or α-methyldopamine given i.p. or intracerebroventricularly. This was prevented by inhibition of central dopamine β-hydroxylase (with 1-phenyl 3-(2-thiazolyl)-2-thiourea) or by intracerebroventricular treatment with α-adrenoceptor blocking agents (phentolamine, tolazoline). The dopamine receptor antagonists (haloperidol, flupentixol) had no effect. These results indicate the following sequence of events within the CNS: α-methyldopa → α-methyldopamine → α-methylnoradrenaline → stimulation of α-adrenoceptors → hypotension (see Section 3.2.).

A decrease in the blood pressure and heart rate of rats followed the microinjection of noradrenaline, adrenaline or α-methylnoradrenaline into the lower brainstem in the area of the nucleus of the solitary tract (*de Jong,* 1974; *de Jong* et al., 1975; *Struyker Boudier* et al., 1975b). Hypotensive responses to electric stimulation or to microinjection of 23 nmol α-methylnoradrenaline were most pronounced when elicited from a common site comprising the middle-caudal part of the nucleus tractus solitarii at the obex level (*de Jong* et al., 1975).

Hoyer and *van Zwieten* (1971; 1972) infused amphetamine and related sympathomimetic amines into the vertebral artery of cats. (±)-Amphetamine (50 or 150 μg/kg) decreased blood pressure (but not heart rate), and this was prevented by prior i.v. treatment with piperoxan, yohimbine or haloperidol (0.6 and 1 mg/kg). Intravenous injection of the same dose of amphetamine increased the blood pressure. Similar results were obtained with (±)-ephedrine, phentermine and chlorphentermine. The authors stated that "central hypotension as a result of stimulation of central α-adrenoceptors seems to be a general principle" (*Hoyer* and *van Zweiten,* 1972).

The intracisternal injection of 10-30 μg/kg of the imidazolines naphazoline, oxymetazoline or St 91 was then shown to produce the whole medullary autonomic response pattern described for the clonidine-like hypotensive drugs (see Section 2.4.2.5.). The decrease in sympathetic cardiovascular tone was first demonstrated by the fall in blood pressure and heart rate in atropine-treated, vagotomized dogs and cats (*Kobinger* and *Pichler,* 1975c) and later by the decrease in splanchnic nerve discharge in cats (*Kobinger* and *Pichler,* 1976). The activation of the vagally mediated baroreceptor reflex was demonstrated in dogs after inhibition of β-adrenoceptors. The intracisternal injection of each of the three imidazolines facilitated the bradycardic response to i.v. injection of angiotensin. This experimental series illustrates the complications which may result from the "leakage" of small amounts of highly active vasopressor drugs from the cisternal spaces into the peripheral circulation. In some of the experiments with naphazoline and oxymetazoline, no facilitation of the reflex bradycardia was observed. However, in these experiments, there was a significant increase in blood pressure which counteracted the reflex response (*Kobinger* and *Pichler,* 1975c). All three imidazolines increased the blood pressure and did not facilitate the baroreceptor reflex when injected i.v. or s.c. In similar experiments, intracisternal injections of naphazoline and oxymetazoline failed to affect blood pressure and sympathetic nerve activity (*Schmitt* and *Fénard,* 1971). So far no explanation has been given for this discrepancy.

These central actions of sympathomimetic amines can be localized in the medulla with a good degree of probability (compare with Section 2.4.2.6.). The following results show that regulation of cardiovascular events might also be mediated by α-adrenoceptors at hypothalamic sites. Hypotension and bradycardia were induced by the microinjection of the following sympathomimetic agents into the anterior hypothalamic/preoptic region of rats: noradrenaline (3-40 nmol), α-methylnoradrenaline (5-15 nmol), adrenaline (0.3-30 nmol), phenylephrine (30-100 nmol), tetrahydrozoline (60 nmol) and St 666 (20 nmol) (*Struyker Boudier* et al., 1974a, 1975a; *Struyker Boudier,* 1975). Dopamine (30 and 70 nmol) and isoprenaline (3 and 12 nmol) were ineffective indicating no involvement of dopamine receptors or β-adrenoceptors (*Struyker Boudier,* 1975). The hypotensive effect of noradrenaline in rats was confined to a very specific region (anterior hypothalamic or preoptic region) and had no effect or even increased the blood pressure at other hypothalamic sites. It was suggested that the pressor responses were due to leakage of noradrenaline into the peripheral circulation since this substance increases blood pressure in i.v. doses 1/300 of those needed intrahypothalamically for the hypotensive effect (*Struyker Boudier* et al., 1974a; *Struyker Boudier,* 1975).

However, there are also reports suggesting that α-adrenoceptors in the hypothalamus might mediate a pressor response (*Philippu* et al., 1971). Perfusion of the third ventricle with noradrenaline or adrenaline (2×10^{-3} *M*) markedly increased the blood pressure of intact cats, and this was diminished after spinal cord section at C 2. Electric stimulation of various parts of the hypothalamus (posterior, ventromedial and anterior medial nuclei) causes a release of noradrenaline and its metabolites into the perfused third ventricle (*Philippu* et al., 1970). α-Adrenoceptors mediating pressor responses were later thought to be localized in the posterior part of the hypothalamus where electric stimulation produced a blood pressure increase which was antagonized by the local superfusion of the stimulated areas (push-pull cannula) with high concentrations of tolazoline (5×10^{-2} *M*) or 10^{-1} *M* piperoxan (*Philippu* et al., 1973).

Thus, α-adrenoceptors which mediate hypotension and bradycardia were detected in medullary and anterior hypothalamic areas whereas α-adrenoceptors which are involved in pressor responses were detected in the posterior hypothalamus. α-Adrenoceptors situated in the spinal medulla inhibit sympathetic preganglionic neurones. The stimulation of these latter receptors by clonidine may add to the overall hypotensive effect of the drug. It is striking that the systemic administration of hypotensive drugs with α-adrenoceptor-stimulating properties (clonidine-like agents and α-methyldopa) have never been reported to increase the discharge of sympathetic nerves. From transection experiments (see Section 2.4.2.6.), it has been postulated that clonidine increases blood pressure by an action on the forebrain, but apparently this effect (if it exists) is masked by the predominant hypotensive effect. It must be concluded, therefore, that central α-adrenoceptors which mediate an increase in blood pressure are either fewer in number or are less accessible from the blood stream than their "opponents," i.e., the central α-adrenoceptors which mediate hypotension. In midbrain-transected animals all the essential cardiovascular and sympathoinhibitory effects of clonidine are seen with the same doses as in intact animals (*Schmitt* and *Schmitt,* 1969; *Kobinger* and *Pichler,* 1975b). Thus, it seems that the bulbar α-adrenoceptors play a more important role in the response to hypotensive drugs than do the suprabulbar α-adrenoceptors.

4.3. Comparison of Central and Peripheral α-Adrenoceptor Effects

The previous section reviewed different α-adrenergic substances which produced cardiovascular depressor effects when injected directly into the brain. It therefore seems to be only a question of penetration from the blood stream to the central α-adrenoceptors which determines whether a substance, shown at peripheral sites to be an α-adrenoceptor agonist, acts as a " central hypotensive" agent. The central action must then be a function of the peripheral α-adrenoceptor effect and a penetration factor. The lipophilicity of a drug is a factor which determines the penetration of a drug into brain tissue. Using a limited number of imidazolines (clonidine, tolonidine, flutonidine, St 91, St 93 and St 608), the relation was determined between the central sympathoinhibitory effect (as measured by the bradycardia following i.v. injection of vagotomized rats) and the product of peripheral α-adrenoceptor potency (as measured by the pressor response in spinal rats) times the lipid affinity (as measured by the partition coefficient of octanol/aqueous buffer, pH 7.4; *Kobinger,* 1974;*Hoefke* et al., 1975). A correlation between the two variables was obtained for five of the six substances (see Fig. 11.) It can be concluded, therefore, that a correlation exists between central cardiovascular depression, peripheral α-adrenoceptor potency and lipid affinity within a group of chemically closely related drugs. St 91 did not fit the curve because of lack of central bradycardic activity after i.v. injection. However, this drug, being a potent peripheral α-adrenoceptor agonist, exerted a clonidine-like cardiovascular activity after intracisternal injection into cats and dogs (*Hoefke* et al., 1975; *Kobinger* and *Pichler,* 1975c). This demonstrated that lipid affinity is not the only factor determining penetration to the area of central "cardiovascular depressor" α-adrenoceptors. Other factors, physicochemical or determined by the conformation of the molecule, may also play a role. Similar conclusions can be drawn from the observation that tramazoline and flutonidine were equihypotensive in rats after i.v. injection but flutonidine was a much weaker peripheral α-adrenoceptor ago-

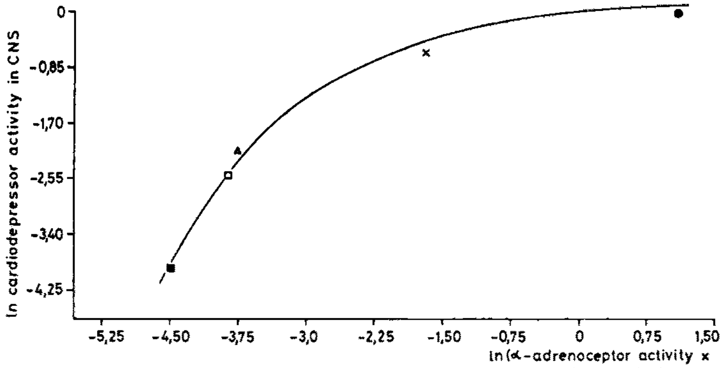


Fig. 11. Relationship between peripheral α-adrenoceptor activity, lipid affinity and centrally mediated cardiodepressor activity. Abscissa: natural logarithm of the product of relative activity on peripheral α-adrenoceptors. This was derived from determination of pressor effects in spinal rats (Section 2.2.2.) multiplied by the partition coefficient of octanol/water. Ordinate: natural logarithm of the relative CNS activity as derived from bradycardic response in vagotomized rats (Section 2.3.2.). Partition coefficients: ● clonidine, 3.0; x St 93, 0.29; △ tolonidine, 0.11; ■ flutonidine, 0.15; □ St 608, 0.27; St 91, 0.06. The latter is not included in the graph because of a lack of central cardiodepressor activity. From *Hoefke* et al. (1975)

nist *and* had less lipid affinity than tramazoline (compare data of *Struyker Boudier* et al., 1974b; and 1975a).

The existance of different types of α-adrenoceptors may also mask the correlation between peripheral and central α-adrenergic effects. Different α-adrenergic drugs have been compared with respect to their peripheral effect (inhibition of contractions of the isolated rabbit intestine) and their central bradycardic effect in rats. The latter was produced by stereotactic injection (1 μl) of drug solutions into the anterior hypothalamic/preoptic region (determination of threshold doses) to avoid as many physicochemical barriers as possible (*Struyker Boudier* et al., 1974b; 1975a). The order of activity differed at the two test sites; e.g., α-methylnoradrenaline was four times more potent than noradrenaline at the central site, but 1/4 as potent in the intestine. The differences were even more striking for the imidazolines: naphazoline, St 91 and especially oxymetazoline were much more potent than clonidine in the peripheral test organ, but were inactive in the hypothalamus. These results led to the conclusion that structural requirements for activation of α-adrenoceptors are different at peripheral and central cardiovascular sites (*Struyker Boudier* et al., 1974b; 1975a). The presence of a - N = bridge between the imidazoline and the phenyl moieties (as in clonidine) was considered important for the central action in contrast to the – C – bridge important for peripheral action (as in oxymetazoline). However, this is not a general rule because oxymetazoline and naphazoline both depressed cardiovascular and sympathetic nerve activity in dogs and cats after intracisternal injection (*Kobinger* and *Pichler,* 1975c; 1976).

A difference between central cardiovascular depressant and peripheral α-adrenoceptors has also been postulated by *Schmitt* and his colleagues since a number of classical α-adrenoceptor antagonists behaved quite differently as antagonists of the sympathoinhibitory and hypotensive effects of clonidine. In cats and dogs, the effect of clonidine on sympathetic discharge was not antagonized by tolazoline, phentolamine or phenoxybenzamine (*Schmitt* and *Schmitt,* 1970), but piperoxan and yohimbine were active in this respect (*Schmitt* et al., 1971; 1973b). Moreover, tolazoline, phentolamine or phenoxybenzamine, after intracisternal injection, prevented the hypotensive action of intracisternally applied clonidine in rats and rabbits, while in dogs only tolazoline or phentolamine were active (*Bogaievsky* et al., 1974). It was concluded that clonidine stimulated central α-adrenoceptors, and that the structures of these receptors may vary from species to species.

Bolme et al. (1974) proposed the existence of adrenaline receptors that mediate the hypotensive and respiratory effects (decrease in rate and increase in depth) of clonidine. This idea is based on the immunohistochemical demonstration of phenylethanolamine-N-methyltransferase (which converts noradrenaline to adrenaline) in a catecholamine-containing neurone system with cell bodies in the reticular formation of the medulla and nerve terminals in restricted regions of the spinal cord and in the brain stem (*Hökfelt* et al., 1973). As adrenaline was also found in various rat brain nuclei, an adrenaline-containing neurone system was postulated (see *Fuxe* et al., 1975). Nerve endings of this system were found in various autonomic centres, e.g., the nucleus tractus solitarii and nuclei of the hypothalamus. The involvement of special adrenaline receptors in vasomotor control was proposed because piperoxan or yohimbine blocked the hypotensive action of clonidine but not the clonidine-induced facilitation of the flexor reflex which is dependent on noradrenaline receptor activity (*Bolme* et al., 1974). This proposal is an interesting variant of the hypothesis of different α-adrenoceptors in the CNS – and at peripheral sites – but further definition of the adrenaline-α-adrenoceptors is necessary.

As indicated by *van Rossum* (1965; see also Section 2.2.2.), different types of α-adrenoceptors seem to exist at different peripheral sites. Analogous differences may be expected at central sites but definite proof will be difficult to obtain because of differences in the penetration of drugs, even after intraventricular or intracerebral injection. This is illustrated by the report of *Struyker Boudier* et al. (1974a) who reported a hypotensive effect of clonidine when microinjections were given into various parts of the hypothalamus. In contrast, noradrenaline acted only in a very restricted area of the anterior hypothalamus. Thus, even after microinjection, clonidine diffuses much better within the brain tissue to reach the hypotensive centers than does noradrenaline.

4.4. Involvement of Central Presynaptic and Postsynaptic α-Adrenoceptors

A number of biochemical effects are produced by clonidine-like drugs (in the CNS or in brain slices) which seem to be mediated by α-adrenoceptors and which ultimately lead to decreased activity within central adrenergic neurones. It has been concluded that "autoreceptors" (i.e., receptors on the pericaryon, dendrites or terminals which are responsive to the transmitter of the neurone; *Svensson* et al., 1975) may stimulate a negative feedback system similar to that of peripheral sympathetic nerve endings (see Section 2.2.3.).

Andén et al. (1970) first reported that clonidine decreased the turnover of noradrenaline in rat brain. Pretreatment with clonidine (30 or 100 μg/kg, i.p.) slowed the rate of disappearance of endogenous noradrenaline after inhibition of tyrosine hydroxylase by α-methyl-p-tyrosine methylester (H 44/68; α-MpT). This effect of clonidine was antagonized by phenoxybenzamine or haloperidol. The authors suggested that the reduced noradrenaline turnover was due to a negative feedback in noradrenaline neurones, secondary to an action of clonidine upon noradrenaline receptors. In analogous experiments, yohimbine, piperoxan and tolazoline were also shown to antagonize this effect of clonidine (*Andén* et al., 1976). Clonidine not only slowed the "utilization" (i.e., the rate of disappearance) of noradrenaline but also decreased the rate of synthesis. After the injection of [^{3}H]-tyrosine, the amount of [^{3}H]-noradrenaline formed was measured in various parts of rat brain. The rate of synthesis was reduced by the i.p. injection of clonidine (50 μg/kg; *Rochette* and *Bralet,* 1975). A decreased synthesis was also evident from the clonidine inhibition of dopa accumulation after inhibition of dopa decarboxylase in the noradrenaline-rich regions of rat CNS and spinal cord. The effect was counteracted by various α-adrenoceptor blocking drugs (*Andén* et al., 1976). The accumulation of dopa in decarboxylase-inhibited rats was markedly reduced caudal to a section of the spinal cord (performed 24 hours earlier) indicating a decreased noradrenaline synthesis. Clonidine did not cause any further decrease and α-adrenoceptor blocking agents did not cause an increase in the accumulation of dopa caudal to the transection. Thus, the α-adrenoceptor-mediated feedback mechanism that modulates noradrenaline synthesis is dependent on nerve impulses (*Grabowska* and *Andén,* 1976).

A decreased turnover of noradrenaline in rat brain was also observed after i.p. injection of 0.1-5 mg guanabenz/kg (*Bolme* et al., 1973) and after oral ingestion of 5 mg BS 100-141/kg (*Saameli* et al., 1975).

Slices of rat cerebrum were preincubated with [^{3}H]-noradrenaline, superfused with buffer solution and subjected to field stimulation. Stimulation performed in this way induces a noradrenaline release due to depolarization and is very similar to direct nerve stimulation (see *Farnebo* and *Hamberger,* 1973). The addition of clonidine (10^{-7} - 10^{-5} *M*) to the perfusate diminished the stimulation-induced tritium overflow. The degree of inhibition was greater at low frequency of stimulation than at high frequency (5 or 10 Hz, respectively; *Farnebo* and *Hamberger,* 1971; *Starke* and *Montel,* 1973). The effect of clonidine was antagonized by phenoxybenzamine and phentolamine and therefore probably involved α-adrenoceptors. Analogous results were obtained with oxymetazoline (10^{-7} - 10^{-5} *M*). This mechanism regulates the amount of transmitter released per stimulus in the CNS, as observed for peripheral adrenergic nerve endings (*Starke* and *Montel,* 1973; see Section 2.2.3.). In these cerebrum slices it is unlikely that intact neurones exist. Therefore, the negative feedback mechanism must operate locally at the nerve ending, either transsynaptically (i.e., postsynaptic receptor → information to corresponding nerve ending) or presynaptically (autoreceptor at the nerve ending). From our knowledge of peripheral adrenergic nerves, the latter hypothesis appears more likely. A change in the central release of noradrenaline in vivo, as a result of α-adrenoceptor stimulation, has been demonstrated by *Braestrup* (1974): clonidine (500 μg/kg) decreased, and several α-adrenoceptor antagonists increased, the level of 3-methoxy-4-hydroxy-phenylglycol (a main metabolite of noradrenaline) in the whole rat brain.

Svensson et al. (1975) recorded the spontaneous firing of single cell units of noradrenaline-containing neurones in the locus coeruleus of rats. Clonidine (6 μg/kg), administered either i.v. or microiontophoretically, inhibited the firing of the cells as did noradrenaline when administered by iontophoresis. This effect was interpreted as being due to activation of noradrenaline autoreceptors on or near noradrenergic cell bodies. The resulting inhibition may account for the decrease in turnover of brain noradrenaline seen after the systemic administration of clonidine (see above). Thus, one mechanism of feedback regulation of noradrenaline synthesis and turnover appears to involve changes in the rate of nerve impulses, while another regulation mechanism controls the transmitter release per nerve impulse.

It has been supposed that the cardiovascular depressor effects of centrally acting antihypertensive drugs are caused by a decreased activity of central adrenergic neurones resulting from activation of adrenergic autoreceptors (*Starke* and *Montel,* 1973). This shall be termed the "backward hypothesis." The hypothesis was supported by biochemical and histofluorescence studies which showed a concentration of adrenergic neurones at those sites in the brain stem which are known as centres of cardiovascular regulation. There is a dense network of noradrenergic nerve endings in the nucleus tractus solitarii, the nucleus dorsalis n. vagi, the reticular formation of the medulla oblongata and the hypothalamus. For the origin, pathways and terminals of these neurones, the reader is referred to the papers by *Fuxe* (1965), *Andén* et al. (1966), *Ungerstedt* (1971) and *Fuxe* et al. (1975). As monoaminergic cell bodies have been found only in the lower brain stem, it may be concluded that many adrenergic terminals within the medulla belong to short neurones. The following arguments were used in favour of the "backward hypothesis." (1) Clonidine has a high affinity ratio for presynaptic/postsynaptic α-adrenoceptors of peripheral receptor systems (*Starke* et al., 1974; see Section 2.2.3.). (2) Low doses of clonidine were necessary to decrease the noradrenaline turnover (i.e., to exert a presynaptic effect) and to lower the blood pressure but high doses were necessary to facilitate the hindlimb flexor reflex in spinal rats, an effect on postsynaptic α-adrenoceptors (*Andén* et al., 1976). (3) Relative potencies of α-adrenoceptor blocking drugs differed when these agents were used to inhibit the biochemical and hypotensive effects of clonidine or when they were used to antagonize the flexor reflex activity and the postsynaptic effects of clonidine in reserpine-pretreated animals (*Andén* et al., 1976). However, these differences in potency of clonidine and of α-adrenoceptor antagonists can also be explained by different affinities of the drugs for postsynaptic α-adrenoceptor at various levels of the CNS (e.g., in the medulla or in the spinal cord. This possibility has been pointed out for various peripheral adrenergic systems (see Section 2.2.2.).

The backward hypothesis assumes intact adrenergic neurones and no effect in the absence of endogenous noradrenaline or during functional impairment of the nerve endings. *Dollery* and *Reid* (1973) injected 6-hydroxydopamine – a drug which leads to neuronal degeneration, particularly of noradrenergic nerve endings – into the cisterna magna of rabbits. A few days later, the hypotensive and bradycardic effect of clonidine was markedly attenuated. They concluded that the effect of clonidine depends on the integrity of central monoaminergic neurones. Conversely, *Haeusler* and *Finch* (1972) and *Finch* (1975) found that clonidine still lowered blood pressure after an injection of 6-hydroxydopamine into the lateral ventricle of renal hypertensive rats. It should be noted that the intracerebro-ventricular administration of 6-hydroxydopamine causes only a partial elimination of noradrenaline (*Chalmers* and *Reid,* 1972). Moreover, the local injection of 6-hydroxydopamine leads to a nonspecific destruction of other, noncatecholaminergic, neurones in the brain (*Butcher* et al., 1974). Therefore, a very complex situation exists after pretreatment with this drug which may account for the contradictory results. Contradictory results have also been obtained with drugs which block the reuptake of noradrenaline, e.g., imipramine, desipramine and other tricyclic antidepressants. The hypotensive effect of clonidine and α-methyldopa (in some experiments) was antagonized by the psychotropic drugs in the experiments of *Reid* et al. (1973) and *van Zwieten* et al. (1975), but was not antagonized in other experiments (*Hoefke* and *Warnke-Sachs,* 1974; *Finch,* 1975). *Van Zwieten* and his colleagues did not consider the antagonism between the tricyclic antidepressant agents and clonidine or α-methyldopa to be due to an inhibition of reuptake at presynaptic sites for the following reason: cocaine (a reuptake inhibitor) was ineffective but iprindol (a tricyclic antidepressant which does not inhibit the reuptake of noradrenaline) was effective as an antagonist of the hypotensive effect of clonidine. In these experiments all drugs were infused into the vertebral artery (*van Zwieten* et al., 1975; *van Zwieten,* 1976). The studies indicate that the antagonism is based on competition at central (postsynaptic) α-adrenoceptors (see *Van Zwieten,* 1975a) and therefore is due to the α-adrenoceptors blocking properties of the antidepressant drugs. Therefore, the reports reviewed so far do not unequivocally indicate whether an intact presynaptic adrenergic nerve ending is necessary for the hypotensive actions of clonidine and α-methyldopa.

The following evidence, however, strongly suggests that endogenous noradrenaline is not required for the central cardiovascular action of these drugs. Pretreatment with reserpine profoundly decreases the brain content of noradrenaline (*Pletscher* et al., 1958). Histofluorescence studies have shown that this depletion also extends to the noradrenergic terminals in the brain (*Fuxe,* 1965). Dogs were pretreated subcutaneously with reserpine (2 mg/kg) and the baroreceptor reflex was elicited by i.v. injections of angiotensin. The animals were treated with a β-adrenoceptor antagonist for comparison with unpretreated animals (see Section 2.4.2.4.). The induced reflex bradycardia is mediated by the efferent vagus nerve and does not involve any adrenergic transmitter at peripheral sites. The intracisternal injection of clonidine (1 μg/kg) to reserpine-pretreated dogs facilitated the reflex by about the same degree as in controls (*Kobinger* and *Walland,* 1973). Phentolamine (0.2 mg/kg, intracisternally) antagonized this effect. These results strongly indicated that those α-adrenoceptors which mediate the central cardiovascular effects of clonidine-like drugs act independently of central endogenous noradrenaline (Fig. 8).

These experiments were repeated later in decerebrate rats. Reserpine was used (7.5 mg/kg, 20h) to deplete the catecholamine stores and, in addition, animals were pretreated with α-methyl-p-tyrosine (250 mg/kg, 4-6h) to inhibit the synthesis of the transmitter. As in dogs, clonidine (30 μg/kg, i.v.) facilitated the vagally mediated baroreceptor reflex in noradrenaline-depleted animals as effectively as in controls (*Kobinger* and *Pichler,* 1974; 1975a). *Haeusler* (1974b) pretreated cats with reserpine (5 mg/kg) and α-methyl-p-tyrosine (2 x 300 mg/kg) and thereby reduced the cerebral noradrenaline content below the threshold of detection (5 ng/g). The intravenous injection of clonidine (30-300 μg/kg) reduced the electric discharge of sympathetic nerves but, for the same effect, a threefold higher dose of clonidine had to be used than in unpretreated animals. Using the same pretreatment schedule with reserpine and α-methyl-p-tyrosine in cats, *Kobinger* and *Pichler* (1976) injected 1 μg clonidine/kg intercisternally and clearly reduced the spontaneous electric activity of splanchnic nerve fibers. This method of administration and the low dose avoided a peripheral pressor response and consequent stimulation of the baroreceptor response (which may also decrease sympathetic activity). Moreover, the intracisternal injection of 30 μg oxymetazoline/kg decreased the electric activity of the splanchinic nerve by the same degree in the pretreated cats and in controls (*Kobinger* and *Pichler,* 1976). Oxymetazoline, like clonidine, has been classified as a preferentially presynaptic α-adrenoceptor agonist at peripheral sites (*Starke* et al., 1975; see Section 2.2.3.).

These results cannot be reconciled with the idea that hypotensive agents stimulate central α-adrenoceptors by decreasing the release of endogenous noradrenaline. They provide strong arguments against the "backward hypothesis." Together, the results suggest that the central cardiovascular action is mediated by the stimualtion of postsynaptic α-adrenoceptors ("effector receptors") eliciting the medullary response pattern described in Section 2.4.2.5. ("foreward hypothesis" see Fig. 9).

A postsynaptic action of clonidine is also responsible for the facilitation of the spinal flexor reflex in rats and for the increased motor activity (which clonidine exerts together with apomorphine) since both effects have been demonstrated in noradrenaline-depleted animals (*Andén* et al., 1970; *Strömbom,* 1976).

These arguments do not exclude the possibility that presynaptic adrenergic effects of clonidine or α-methyldopa (via α-methylnoradrenaline) modify the cardiovascular responses to these drugs in some, hitherto undetermined, way.

5. Possible Role of Noradrenaline as a Central Neurotransmitter in Cardiovascular Regulation

The effects of α-adrenoceptor-stimulating hypotensive drugs on the sympathetic as well as the vagal system suggest that these drugs only mimic the effects of the natural transmitter, noradrenaline. Under physiologic conditions, liberation of endogenous noradrenaline at certain central nerve endings might be expected to produce signs of cardiovascular depression and trigger the pattern of autonomic responses described for the hypotensive drugs in Section 2.4.2.5. and in Figure 9. This idea is supported by the rich supply of adrenergic nerve endings in those parts of the medulla which form the cardiovascular or vasomotor centers (see *Fuxe,* 1965 and the preceding section).

One of the physiologic conditions resulting in cardiovascular depression is the baroreceptor reflex. In the afferent arc of the reflex (mainly aortic nerve, carotid sinus nerve), information from baroreceptors reaches the nucleus tractus solitarii as a first relay station (*Humphrey,* 1967; *Crill* and *Reis,* 1968; *Lipski* et al., 1975). Sets of neurones that form anatomically and functionally distinct synapses and that function under electric stimulation as medullary depressor centres (*Alexander,* 1946), conduct the baroreceptor information to vagal neurones (which are facilitated) and to sympathetic neurones (which are inhibited). As clonidine facilitates centrally the cardiodepressor baroreceptor reflex (*Kobinger* and *Walland,* 1971; 1972a; see Section 2.4.2.4.), it was assumed, as a working hypothesis, that the drug mimics the action of endogenous noradrenaline. Hence, noradrenaline might function as an essential link within the central part of the baroreceptor reflex loop. To investigate this possibility, the baroreceptors were stimulated by increasing the blood pressure of dogs with i.v. injections of angiotensin, and the resulting bradycardia was recorded (*Kobinger* and *Walland,* 1973). The animals were pretreated with a β-adrenoceptor blocking drug so that only the vagal part of the reflex was under observation. No adrenergic transmitter is then involved in the peripheral part of the reflex. The first results supported the hypothesis that noradrenaline is an essential transmitter. The α-adrenoceptor blocking agent, phentolamine, given either i.v. (5 mg/kg) or intracisternally (0.2-0.5 mg/kg), practically abolished the cardiodepressor reflex (Fig. 12B). The hypothesis, however, had to be discarded on the basis of the following results: endogenous catecholamine stores were depleted in

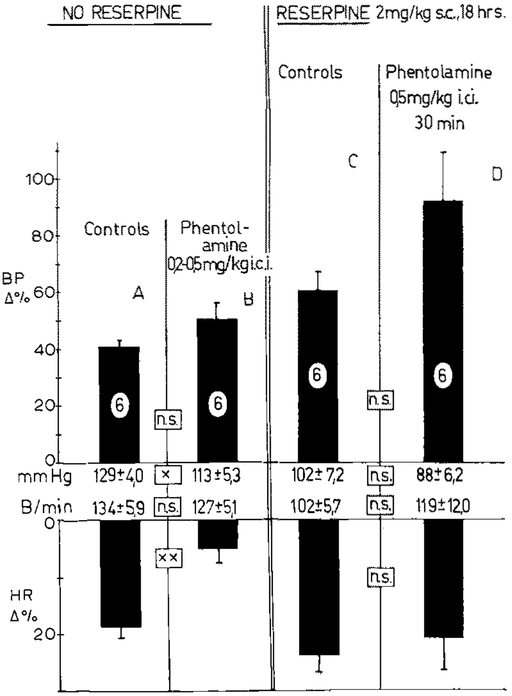


Fig. 12. Effect of phentolamine on the baroreceptor reflex. (Anesthetized dog with spontaneous respiration and β-adrenoceptor blockade with toliprolol). Mean blood pressure and mean heart rate (B = beats) are given between the columns (± S.E.M.).The baroreceptor reflex was elicited by i.v. injections of angiotensin (0.025-0.3 μg/kg). The resulting maximal changes are expressed as the % of the values before angiotensin and are given by the columns as mean ± S.E.M. BP = blood pressure, HR = heart rate. In B and D, angiotensin was given 30 min after intracisternal injection of phentolamine. Numbers of dogs are indicated within the columns. The significance of differences is indicated between those groups which have been compared: xx, $p < 0.01$; x, $p < 0.05$; n.s., not significant ($p > 0.05$). In C and D, dogs were pretreated with reserpine (2 mg/kg, s.c.) 18 h before the experiment. From *Kobinger* and *Walland* (1973)

dogs by pretreatment with reserpine (2 mg/kg, 18h) and the animals exhibited very pronounced reflex bradycardia (Fig. 12C). This result excluded noradrenaline as a transmitter in the reflex arc, especially since no blocking effect was demonstrated with phentolamine (Fig. 12D). However, in spite of pretreatment with reserpine, clonidine (1 μg/kg, intracisternally) facilitated the reflex bradycardia as much as in controls. The facilitation was antagonized by phentolamine (Fig. 8E, F). Essentially similar results were obtained in a later study in decerebrate rats (midbrain transection) pretreated with reserpine and α-methyl-p-tyrosine to deplete stores and inhibit the synthesis of chatecholamines (*Kobinger* and *Pichler*, 1974, 1975a). From these data the following conclusions were drawn. (1) Endogenous noradrenaline cannot be an essential neurotransmitter in the central part of the baroreflex arc.

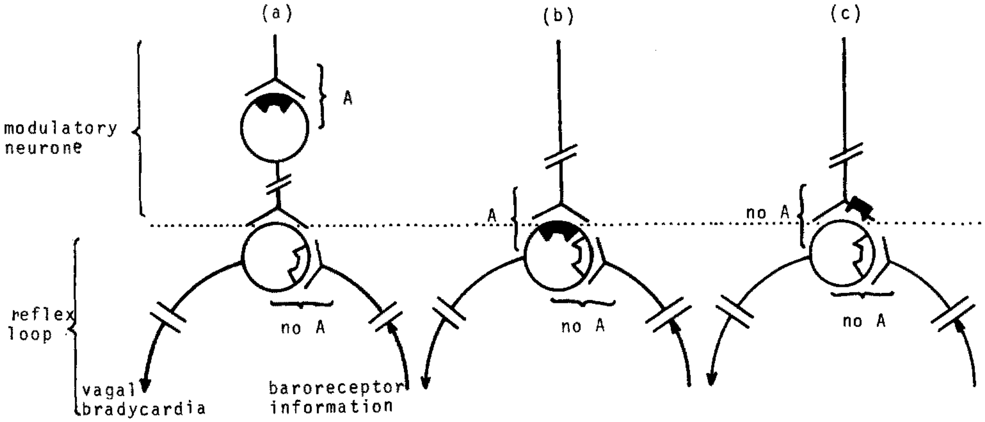


Fig. 13a-c. Schematic representation of the central, medullary part of the baroreceptor reflex loop. No adrenergic link (no A) seems necessary for transmission in the reflex loop as bradycardia can be elicited in spite of depletion of endogenous noradrenaline. Adrenoceptors may be incorporated in an addtional neurone (or chain of neurones) which has a facilitatory influence upon the reflex loop (a), as an additional receptor at one of the neurones of the reflex loop (b) or as a presynaptic inhibitory receptor at a modulatory nerve ending which (by means of a nonadrenergic transmitter) exerts a tonic inhibition upon the reflex (c). Under physiologic conditions, noradrenaline acts as a modulating transmitter (A) in (a) and (b)

Symbols: neurone with cell body, axon and nerve ending; receptor; adrenoceptor; the pathway may be polysynaptic

(2) The α-adrenoceptor which mediates the facilitatory effect of clonidine on the reflex must be localized on a neurone that modulates neurotransmission. Figure 13 shows schematically the possibilities for modulation. Figure 13c presents a presynaptic interference with the reflex transmission. The transmitter(s) which is (are) essential for the reflex arc are not yet known but the results, obtained with animals pretreated with reserpine and α-methyl-p-tyrosine, exclude dopamine and do not favor 5-HT. The observation (see above) that phentolamine is ineffective in reserpine-pretreated dogs but inhibits the reflex in control animals deserves an explanation. Controls received a higher dose of barbiturate and had weaker reflex responses than reserpine-pretreated animals. The response of controls depends on endogenous adrenergic facilitation (blocked by phentolamine). In contrast, the response of pretreated animals, where the reflex acts independently of endogenous adrenergic activities, depends on the nonadrenergic transmitter only (Fig. 13). Thus, it can be concluded that the facilitatory modulation of the reflex, via α-adrenoceptors (as stimulated by clonidine), is operated physiologically by noradrenaline liberated from adrenergic nerve endings which are numerous within the medulla.

Haeusler and *Lewis* (1975) electrically stimulated both sinus nerves of cats and recorded a decrease in electric activity of sympathetic nerves in control and in noradrenaline-depleted animals (pretreatment with reserpine and α-methyl-p-tyrosine). The authors concluded that central adrenergic neurones are unlikely to be an integral part of the baroreceptor reflex. In 1960, *Iggo* and *Vogt* had already reported that pretreatment of cats with reserpine did not attenuate the response of sympathetic nerve discharge to adrenaline or to asphyxia. They concluded that central stores of noradrenaline, dopamine or 5-HT are not essential for normal responses of the autonomic centers. However, these authors carefully indicated that newly synthesized amines might be responsible for central activity. This reservation can be met today on the basis of the experiments cited above where storage *and* synthesis of noradrenaline and dopamine were inhibited. Thus, these two amines do not have an essential transmitter function in the CNS with respect to the cardiovascular stabilizing mechanisms reviewed above.

In variance with these findings and conclusions, *Chalmers* and *Reid* (1972) produced neurogenic hypertension and tachycardia in rabbits by section of afferent baroreceptor sino-aortic nerves. Intracisternal pretreatment of the animals with 6-hydroxydopamine (600 μg/kg) prevented the development of hypertension. Treatment with 6-hydroxydopamine decreased to control levels an already established high blood pressure and heart rate. The results were explained by the existence of central noradrenergic neurones, at the bulbospinal level, which form an essential part of the baroreceptor reflex arc. *Doba* and *Reis* (1974) reached similar conclusions after producing hypertension in rats by lesions in the nucleus tractus solitarii. *Chalmers* and *Reid* (1972) found a decrease in the heart rate after intracisternal administration of 6-hydroxydopamine in normal and neurogenic hypertensive rabbits. They explained this by the exclusion of the central adrenergic influences that normally inhibit the vagus (*Chalmers* and *Reid,* 1972). This conclusion is quite different from the idea reviewed above of the central adrenergic facilitation of the cardiac vagus.

In contrast to *Chalmers* and *Reid* (1972), after intracisternal administration of 6-hydroxydopamine to rabbits, *Haeusler* and *Lewis* (1975) did not find a decrease in baroreflex sensitivity as measured by the reflex response of heart rate to changes in blood pressure. The authors rejected the hypothesis of an adrenergic link in the baroreceptor reflex pathway. The contradictory results obtained after local treatment with 6-hydroxydopamine might be explained by unspecific central neuronal damage (*Butcher* et al., 1974). Moreover, there is great variability in the degree of 6-hydroxydopamine-induced destruction of noradrenergic neurones in different areas of the CNS. Following the intracisternal injection of 6-hydroxydopamine (600 μg/kg), the noradrenaline concentration of the medulla-pons region of the rabbit was reduced to only 61% and that of the spinal cord to 18% (*Chalmers* and *Reid,* 1972).

Electric stimulation of the posterior hypothalamus both increased blood pressure and sympathetic activity and caused a release of noradrenaline into the perfused third ventricle (*Philippu* et al., 1970; 1973). This suggested that endogenous noradrenaline is a transmitter of the pressor messages from the higher brain stem. However, as in medullary regions, the function of noradrenaline as an essential transmitter could not be verified. After pretreatment of cats with reserpine and α-methyl-p-tyrosine, electric activity in sympathetic nerves was increased by stimulation of the posterior hypothalamus as much as in unpretreated controls (*Haeusler,* 1975).

One further argument suggests that adrenergic nerve endings are in a strategic position such that their noradrenaline can reach those adrenoreceptors which mediate cardiovascular depressor functions. After inhibition of peripheral dopa decarboxylase, the amino acids *L*-dopa and *DL*-m-tyrosine lower blood pressure by an action of the CNS. The effects of these two substances are dependent on the presence of endogenous noradrenaline and it has been concluded that they are due to the liberation of noradrenaline in the CNS (*Rubenson* 1971a; b; *Andén* et al., 1972). Obviously, the noradrenaline stores involved in this response cannot be far away from the α-adrenoceptors which mediate the cardiovascular depression.

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Structure and Function of Nuclear Ribonucleoprotein Complexes

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Contents

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Nonstandard Abbreviations

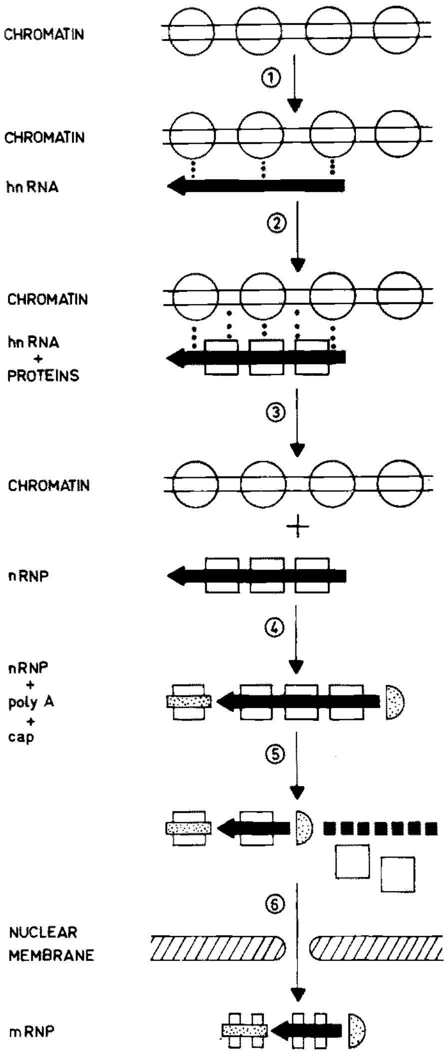
hnRNA	heterogeneous nuclear RNA
nRNP	nuclear ribonucleoprotein

1. Introduction

In prokaryotes, transcription and translation of mRNA are closely linked processes. Large portions of the mRNA are translated before the transcription is completed. The mRNA has a very short half-life and is degraded immediately after translation. The control of protein synthesis is therefore exerted mainly at the level of transcription.

In contrast, the regulation of mRNA formation and of protein synthesis in eukaryotes are more complex processes. The flow of information from the genes in the cell nucleus to the protein-synthesizing machinery in the cytoplasm involves a multitude of molecular components and can be regulated at many steps. The common view of the processes involved in the formation of mRNA is presented schematically in Figure 1. Chromatin is first transcribed in a reaction catalyzed by DNA-dependent RNA polymerase (step 1). The newly synthesized RNA is of high molecular weight and heterogeneous in size, and is therefore named heterogeneous nuclear RNA (hnRNA). Its base composition is roughly DNA-like, so that the name DNA-like RNA (dRNA) is used as well. HnRNA contains intermediate repetitive and unique ("nonrepetitive") sequences which are interspersed with one another. Furthermore, two or three short oligo (U) tracts of about 30 nucleotides in length and oligo (A) tracts of similar size have been found per molecule of hnRNA. HnRNA does not simply consist of a linear RNA strand, but has a secondary structure, mainly due to double-stranded regions. There is evidence from histochemical as well as from biochemical studies that the newly synthesized hnRNA is complexed with nuclear proteins (step 2). The resulting ribonucleoprotein complex dissociates from the chromatin template (step 3) and is posttranscriptionally modified or "processed." At the 3'-end, a poly (A) tract is added by the sequential addition of approximately 200 adenylate moieties in a process independent of a DNA template. At the 5'-terminal sequence, either pppXp, ppXp, pXp are found or a "capping" takes place, i.e. 7-methyl-guanosine is linked through its 5'-hydroxyl group, via a tri- (or di-) phosphate group, to the 5'-hydroxyl group of a 2'-methylated nucleoside (step 4). The modified ribonucleoproteins are then cleaved into smaller fragments by endo- and exonucleolytic enzymes (step 5). The great majority of the hnRNA is broken down in the nucleus and never reaches the cytoplasm. The "trimmed" hnRNA with

Fig. 1. Schematic representation of the various steps involved in eukaryotic mRNA formation



adjacent poly (A) is translocated to the cytoplasm (step 6). In the cytosol, the mRNA is found in a complex with proteins (mRNP). It is not known to what extent the mRNP proteins are indentical with those associated with hnRNA in the nucleus.

Since hnRNA is always complexed with proteins (Fig. 1), a full understanding of mRNA formation requires an appreciation of the role of ribonucleoprotein complexes. In this review, the formation, structure, and function of the primary transcription products, the complexes of hnRNA and proteins which can be isolated as nuclear ribonucleoprotein particles (nRNP particles), will be discussed. Work on the preribosomal RNA-protein complexes will not be dealt with here, having recently been reviewed elsewhere (*Prestayko* et al., 1974; *Kumar* and *Warner*, 1972; *Perry*, 1976; *Maden*, 1976; *Hadjiolov* and *Nikolaev*, 1976)

2. Methods of Isolation

There are several methods available for the preparation of nRNP particles. All start with purified nuclei from which the nRNP particles are extracted and further purified by sucrose gradient centrifugation. The different basic procedures of extraction are summarized in Table 1. In principle, nRNP particles are extracted either from intact nuclei or from homogenates after disruption of nuclei.

The first method for the preparation of nRNP particles was described by *Samarina* et al. (1968), who extracted intact nuclei with isotonic salt buffer, pH 7.0, to remove preribosomes and subsequently several times with the same buffer at pH 8.0. The pH 8.0 extracts contained the nRNP particles. Large amounts of cytosolic proteins including RNAase inhibitor(s) were added to the extraction buffers.

Table 1. Methods of preparation of nRNP particles

1. Extraction from intact nuclei:	
1.1. With an isotonic salt buffer, pH 8.0 in the presence of cytosolic RNAase inhibitor ("diffusion method")	*Samarina* et al. (1968)
1.2. With 0.88 *M* sucrose buffer, pH 7.6 in the presence of cytosolic RNAase inhibitor and ATP at 20°C	*Ishikawa* et al. (1969)
2. Extraction from nuclei disrupted by:	
2.1. Hypotonic Tris-buffer, pH 7.4	*Moulé* and *Chauveau* (1968); *Raj* et al. (1975)
2.2. Sonication in low salt (0.01 *M* NaCl) buffer, pH 7.0	*Bhorjee* and *Pederson* (1973); *Pederson* (1974a)
2.3. Sonication in isotonic (0.1 - 0.14 *M* NaCl) buffer, pH 8.0, with or without cytosolic RNAase inhibitor	*Stevenin* and *Jacob* (1974); *Louis* and *Sekeris* (1976)
2.4. Sonication in 0.35 *M* sucrose, low salt (0.07 *M* KCl) buffer, pH 7.6	*McCarty* et al. (1966)
2.5. Use of 0.2% sodium deoxycholate in isotonic salt buffer, pH 8.0, in the presence of cytosolic RNAase inhibitor	*Stevenin* et al. (1970) *Stevenin* and *Jacob* (1972)
2.6. Dounce homogenization after incubation with DNAase in high salt (0.8 *M* NaCl) buffer, pH 7.4	*Faiferman* and *Pogo* (1975)
2.7. Use of a French press in 0.25 *M* sucrose buffer, pH 7.6, with or without cytosolic RNAase inhibitor	*Faiferman* et al. (1970)
3. Extraction from chromatin by incubation with 0.25 *M* sucrose buffer, pH 7.6 at 20°C	*Ishikawa* et al. (1974)

The methods for the preparation of nRNP particles developed afterwards involved disruption of nuclei, achieved by osmotic shock, sonication, detergents, or mechanical forces (Dounce homogenizer, French press). It is not possible to compare the yields of nRNP particles in terms of RNA and protein obtained according to the different preparation methods. However, various authors found that 30% - 40% of the rapidly labeled RNA could be extracted from cell nuclei (*Faiferman* et al., 1970; *Augenlicht* and *Lipkin,* 1976; *Gross* et al., 1977).

The main problem in the preparation of nRNP particles is the separation of different contaminants. Possible candidates are chromosomal proteins, preribosomal particles, nuclear sap proteins, ribosomal, and cytosolic proteins. To minimize the contamination of nRNP particles by ribosomal and cytosolic proteins, pure nuclei have to be used. Ribosomes attached to the outer nuclear membrane and intranuclear ribosomal subunits are effectively removed by washing nuclei with EDTA (*Pederson,* 1974a; *Louis* and *Sekeris,* 1976) or by the use of detergents such as triton X-100 (*Faiferman* and *Pogo,* 1975). Although nRNP particles were prepared by lysis of nuclei with 0.2% of sodium deoxycholate (*Stevenin* and *Jacob*, 1972), higher concentrations of the detergent are unfavorable for the stability of the particles (*Moulé* and *Chauveau,* 1968, *Faiferman* et al., 1971; *Stevenin* and *Jacob,* 1972; *Stevenin* et al., 1975). Similarly, treatment of the nuclei with triton X-100 resulted in an increased degradation of nRNP particle RNA, probably by activation of latent RNAases (*Lund-Larsen,* 1975). When particles were isolated from sonicated nuclei in the presence of different salt concentrations, various amounts of contaminants could be found. Whereas *Pederson* (1974 a) did not detect chromosomal proteins upon extraction of HeLa cell nuclei with 0.01 *M* NaCl, *Augenlicht* and *Lipkin* (1976) found in HT 29 cells a ratio of DNA to RNA in nRNP particles of 0.6, indicating a contamination with chromatin. The use of NaCl at concentrations $\geqslant$ 0.3 *M* during the extraction on nRNP particles resulted in a considerable contamination, mainly with histones (*Pederson,* 1974 a; *Northemann* et al., 1978). High salt concentrations as used by *Faiferman* and *Pogo* (1975) in their high salt (0.8 *M* NaCl)-buffer - DNAase method led to a marked loss of particle proteins, in particular in the molecular weight range of about 40,000 daltons. This was also observed by *Gallinaro-Matringe* et al. (1975) who dissociated proteins from rat brain nRNP particles with 0.7 *M* NaCl, but even at a concentration of 0.25 *M* NaCl protein could be dissociated. The lowest contamination of nRNP particles with chromatin was obtained when the extraction was carried out with isotonic salt-buffer solutions (*Pederson,* 1974; *Gallinaro-Matringe* et al., 1975). *Northemann* et al. (1978) determined approximately 5% of DNA and less than 1% of histones related to the particle RNA or to the particle proteins, respectively.

Table 2. Properties of nRNP particles

References	System	Method of preparation[a]	Size of nRNP particles[b] (S value)	Density (g/ml)	Protein to RNA ratio	Number of proteins	Size of RNA (S value)
Samarina et al. (1968)	Rat liver	1.1. (+)	60 - 80	1.40	4	20 - 40	8.5 - 32
Lukanidin et al. (1971, 1972)			(up to 200)			(identic proteins)	
Schweiger & Hannig (1968)	Rat liver	1.1. (-)	30			3 - 4	
Niessing & Sekeris (1971a)	Rat liver	1.1. (+)	130 - 230 (up to 400)	1.39 - 1.40	4	14	
Sekeris & Niessing (1975)	Rat liver	1.1. (+)		1.40	6.2		5 - 80
Yoshida & Holoubek (1976)	Rat liver	1.1. (-)	30	1.397		28	
Patel & Holoubek (1976)							
Ishikawa et al. (1969)	Rat liver	1.2. (+)	45 - 60	1.40	5.6		4 - 28
Smuckler & Koplitz (1974)		1.2. (+)	35 - 45				20-30
Moulé & Chauveau (1968)	Rat liver	2.1. (-)	40		8		3.4
Pederson (1974b)	Rat liver	2.2 (-)	40				
Tata & Baker (1975)	Rat liver	2.2. (-)					30
Albrecht & van Zyl (1973)	Rat liver	2.3. (-)				20	
Louis & Sekeris (1976)	Rat liver	2.3. (-)	30 - 45	1.40			
Northemann et al. (1977, 1978)	Rat liver	2.3. (-)	38	1.39	7 - 8	45 - 50	4 - 8
		2.3. (+)	(up to 200)	1.39	4 - 6	45 - 50	4 - 8, 15 - 20
McCarty et al. (1966)	Rat liver	2.4. (-)	45	1.41 - 1.45			6 - 10
Parsons & McCarty (1968)							
Faiferman & Pogo (1975)	Rat liver	2.6. (-)	43	1.40	4.5		21 - 22
Faiferman et al. (1970)	Rat liver	2.7. (+)	60	1.38 - 1.41	4		10 - 60
Faiferman et al. (1971)			(up to 120)				
Ishikawa et al. (1974)	Rat liver	3.1. (-)	45 (up to 80)	1.40	4		16
Sauermann (1976)	Rat liver	release[c]	20 - 30 (up to 45)	1.43			10 - 15
Martin & McCarthy (1972)	Mouse liver	1.1. (+)	30 (up to 120)	1.39 - 1.41	4		
Stevenin et al. (1970)	Rat brain	2.5. (+)	75 - 110 (up to 300)	1.40	7	45	5 - 9 10 - 65

References	System	Method of preparation[a]	Size of nRNP particles[b] (S value)	Density (g/ml)	Protein to RNA ratio	Number of proteins	Size of RNA (S value)
Gallinaro-Matringe et al. (1975) *Stevenin* et al. (1976) *Knowler* (1976)	Rat uterus	1.1. (+)	30	1.41 - 1.43			
Liao et al. (1973)	Rat prostate	2.1. (-)	30				
Liang & Liao (1974)	Calf uterus	2.1. (-)	50				
Albrecht & van Zyl (1973)	Hepatoma	2.3. (-)				20	
Pederson (1974a)	HeLa cells	2.2. (-)	76 (up to 250)	1.43 - 1.45		12 - 25	20 - 60
Liautard et al. (1976)	HeLa cells	2.2. (-)	85 - 130 (up to 200)				
Ducamp & Jeanteur (1973)	HeLa cells	2.5. (-)	30 - 40	1.39 - 1.41			9 - 12
		2.3. (-)	30 - 40				
Augenlicht & Lipkin (1976) *Augenlicht* et al. (1976)	HT 29 human colon carcinoma cells	2.2. (-)	76 (up to 200)	1.43	4		15
Martin & McCarthy (1972) *Quinlan* et al. (1974)	Mouse ascites	1.1. (+)	30 (up to 80)	1.39 - 1.41	4		
Firtel & Pederson (1975)	Slime mold	2.3. (-)	55	1.41 - 1.43			15
Alfageme & Infante (1975)	Sea urchin embryo	CsCl (4 M)[d]		1.4 - 1.55	4		10 - 35
Ajtkhozhin et al. (1975)	Wheat embryo	2.5. (-)		1.4			15 - 30

[a] The numbers given refer to Table 1; preparation carried out in the presence (+) or absence (-) of cytosolic RNAase inhibitor.

[b] The numbers given refer to the maximum in the nRNP particle sedimentation profile, the numbers in parenthesis represent the sedimentation coefficients of the largest particles.

[c] Immobilized nuclei were perfused with 0.25 *M* sucrose buffer, pH 7.4.

[d] Nuclei were homogenized in 4 *M* CsCl, 0.01 *M* Tris buffer, pH 7.4.

With the various basic preparation methods mentioned above, nRNP particles have been isolated from different tissues or cell lines by various workers. Table 2 gives a survey of the studies on the properties of isolated nRNP particles. It can be seen that most authors found protein to RNA ratios of 4 in the nRNP particles, but higher protein to RNA ratios were also observed, in particular in rat liver and rat brain. The densities of the various particle preparations determined in CsCl gradients are in the range of 1.4 g/ml. In this respect, the densities and protein to RNA ratios do not show a correlation in all cases. This discrepancy could be due to the different methods used by the various authors for the determination of the particle components. The protein to RNA ratios were determined either by calculating the composition from the density of the particles, or from the ratio of absorbance at 260 and 280 nm. The most reliable data on the particle composition are probably those obtained from independent determinations of protein and RNA. Although *Samarina* et al. (1968) have described only one protein species within the nRNP particles, there is now general agreement among various authors that many proteins (up to 45) of different molecular weights are associated with the particle RNA. The size of the particle RNA has been determined by sucrose density gradient centrifugation and polyacrylamide gel electrophoresis. It can be seen from Table 2 that a great heterogeneity in size exists. There is, however, a correlation between the size of the RNA and the sedimentation coefficient of the nRNP particles. The size of the nRNP particles as measured by their sedimentation coefficients depends on the tissue used as starting material and on the methods of preparation. Nuclear ribonucleoprotein particles of high molecular weight are isolated after addition of a cytosolic RNAase inhibitor or by using tissues which have low endogeneous nuclear RNAase activities. In general, neoplastic tissues show very low RNAase activities (*Daoust* and *Lamirande,* 1975). Low RNAase activities have also been observed in rat brain (*Munoz* and *Mandel,* 1968).

3. Structural Aspects of Nuclear Ribonucleoprotein Particles

3.1. Mono-, Polyparticles

Samarina et al. (1968) have shown that mild RNAase digestion of high molecular weight nRNP particles from rat liver leads to the formation of a uniform species of 30 S particles. Similar observations were made by *Stevenin* et al. (1970) using 80 S – 300 S particles from rat brain. Mild digestion with pancreatic RNAase resulted in the formation of 40 S – 50 S particles. *Pederson* (1974 a) converted particles of up to 250 S from HeLa cells into a single species of 45 S structures with pancreatic RNAase. These experiments suggested a polymeric structure of the particles designated as "polyparticles" which can be transformed into more stable units of 30 S – 50 S designated as "monoparticles." From these findings it can be concluded that the low molecular weight particles isolated by various authors (Table 2) are the result of degradation by endogeneous RNAases. The polyparticles probably represent a more native form of nRNP complexes than the monoparticles. Cytosolic RNAase inhibitor has therefore been used by many investigators. The influence of various amounts of cytosolic RNAase inhibitor used during the isolation of nRNP particles on the yield and size of particles from rat liver has been studied by *Faiferman* et al. (1970). In the presence of inhibitor they observed an increase in the yield of all particles, mainly in the range of 80 S – 120 S. *Louis* and *Sekeris* (1976) found that in the presence of RNAase inhibitor polymer structures could be isolated. Similar results were obtained by *Gross* et al. (1977) with rat liver as shown in Figure 2. Increasing amounts of RNAase inhibitor led to a higher yield and a shift in the sedimentation profiles of nRNP particles toward higher molecular weights. On the other hand, *Knowler* (1976) found that the use of rat liver RNAase inhibitor during the extraction of the particles from rat uteri only slightly improved the yield but did not result in heavier particles. In tissues with low RNAase activities such as HeLa cells (*Pederson,* 1974 a; *Liautard* et al., 1976) and HT 29 human colon carcinoma cells (*Augenlicht* and *Lipkin,* 1976; *Augenlicht* et al.,

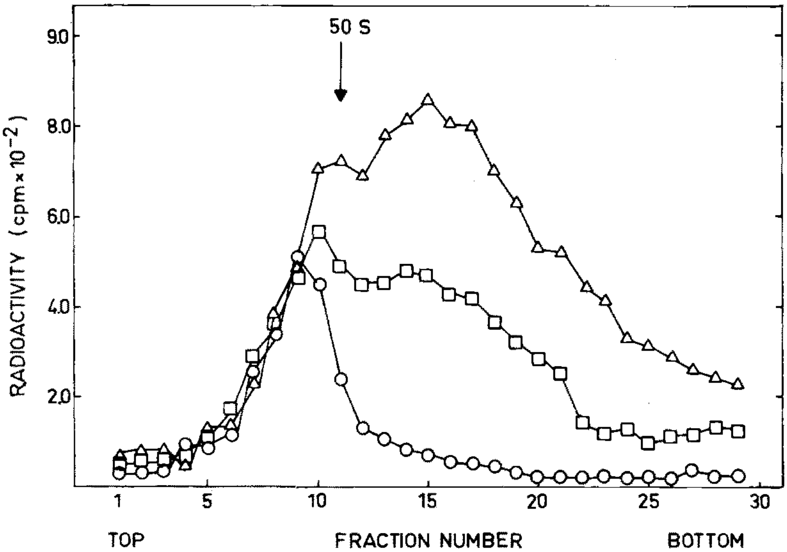


Fig. 2. Effect of cytosolic RNAase inhibitor on yield and size of nRNP particles from rat liver. Nuclear RNP particles extracted from nuclei either in the absence (○ – ○) or in the presence of 300 (□ – □) and 1000 (△ – △) units of cytosolic RNAase inhibitor were subjected to sucrose gradient centrifugation (*Gross* et al., 1977)

1976), polymer structures are obtained even in the absence of RNAase inhibitor. In the case of rat liver, it seems to be difficult to prepare polyparticles in the absence of RNAase inhibitor, indicating high nuclear RNAase activities. *Scheurlen* et al. (unpubl. results) have demonstrated the presence of two RNAase activities in rat liver nuclei. It is shown in Figure 3 that these RNAase activities cannot entirely be separated from the nRNP particles during sucrose gradient centrifugation.

Although the cytosolic RNAase inhibitor is very useful for the preparation of polyparticles, there are problems connected with its use . Because the RNAase inhibitor from rat liver is extremely labile (*Roth,* 1958; *Shortman,* 1961; *Gribnau* et al., 1969; *Gribnau* et al., 1970), a routine purification of the inhibitor to homogeneity is hardly feasible, so that an only partially purified preparation containing cytosolic proteins and low molecular weight cytosolic RNA has to be used. Both contaminants may interfere with the analysis of the components of the particles. Pure synthetic RNAase inhibitors are therefore desirable. A number of RNAase inhibitors such as polyvinyl sulfate, bentonite, macaloid, diethylpyrocarbonate, spermine, and *N*-ethylmaleimide have been described. *Herman* et al. (1976) used a mixture of polyvinyl sulfate, spermine, and *N*-ethylmaleimide to inhibit nuclear RNAases during the isolation of high molecular weight RNA from HeLa cell nuclei. When the nRNP particles were prepared from rat liver in the presence of polyvinyl sulfate or spermine, only incomplete inhibition of RNAase activity could be demonstrated (*Northemann* et al., 1978). The use of the inhibitors had virtually no influence on the yield and size of the particles. Bentonite was found to be a potent RNAase inhibitor, but it adsorbed not only the RNAases but also the nRNP particles (*Northemann* et al., 1978).

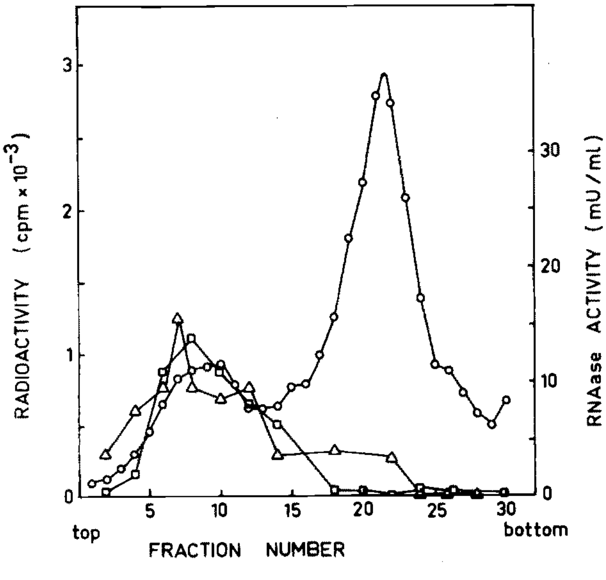


Fig. 3. Distribution of poly (A) and high molecular weight yeast RNA degrading activites after sucrose gradient centrifugation of a sonicated nuclear extract. TCA-precipitable radioactivity (○), poly (A) – (□) and RNA-(△) degrading enzyme activities (*Scheurlen* et al., unpublished results)

3.2. Properties of the RNA

Most studies on the structure and function of hnRNA have been carried out with protein-free RNA. Although many structural features have been worked out, there is presently no clear picture of the structure of hnRNA. The high molecular weight hnRNA is heterogeneous in size, possesses a DNA-like base composition, and shows a high rate of turnover. The presence of mRNA sequences within the hnRNA has been demonstrated. At the 3'-end, a poly (A) tract is added after transcription in a process independent of a DNA template. At the 5'-terminal sequence, a "cap" (*Rottman* et al., 1974) can be formed by the condensation of a guanylate residue in a 5'-5 triphosphate linkage and the methylation of two or three 5'-terminal nucleotides. In addition, ppXp- and pXp-structures are also found (*Schibler* and *Perry,* 1976; *Salditt-Georgieff* et al., 1976; *Bajszár* et al., 1976). Furthermore, internal oligo (A) and oligo (U) tracts which apparently are transcribed from the DNA template are known (*Nakazato* et al., 1973 and 1974; *Burdon* and *Shenkin,* 1972; *Molloy* et al., 1972; *Dubroff* and *Nemer,* 1975; *Korwek* et al., 1976). There are repetitive sequences in hnRNA which may be involved in double-strand formation (*Ryskov* et al., 1972; *Jelinek* and *Darnell,* 1972). These important contributions to the structure and function of hnRNA have been summarized in several reviews which have recently appeared (*Mathews,* 1973; *Darnell* et al., 1973; *Weinberg,* 1973; *Brawerman,* 1974; *Darnell,* 1975; *Lewin,* 1975; *Burdon,* 1976; *Molloy* and *Puckett,* 1976; *Perry,* 1976).

Since the primary gene transcripts are never found as naked RNA in the nucleus, but rather associated with proteins, the native structure and function of newly synthesized RNA can only be understood when nRNP particles are studied. Many authors have examined the RNA of the isolated nRNP particles by sucrose gradient centrifugation or polyacrylamide gel electrophoresis (Table 2). In all cases it was found that the RNA is highly polydisperse. *Samarina* et al. (1968) described for rat liver a correlation between the S values of particles and of RNA isolated from them by analyzing the sedimentation properties of RNA isolated from various zones of a sucrose gradient containing nRNP particles of different sizes. The 30 S, 45 S, 60 S, 75 S and 120 S - 130 S particles contained 8.5 S, 14 S, 17 S, 21 S, and 32 S RNA, respectively. *Pederson* (1974a) found 20 S - 60 S RNA in 40 S - 250 S nRNP particles from HeLa cells. Using HT 29 cells, *Augenlicht* and *Lipkin* (1976) isolated 15 S RNA from nRNP particles of about 76 S. *Niessing* and *Sekeris* (1971a) found a highly heterogeneous RNA of 5 S - 80 S in rat liver nRNP particles of 30 S - 400 S. *Ducamp* and *Jeanteur* (1973) prepared from HeLa cells nRNP particles with S values of 30 - 60. The isolated RNA ranged between 9 S and 12 S.

It is well known that the RNA within the nRNP particles is rapidly labeled with radioactive RNA precursors, for example orotic acid or uridine, indicating a high turnover rate of the particle RNA. *Sekeris* and *Niessing* (1975) presented evidence for the existence of two different RNA species in 30 S nRNP particles of rat liver. By double labeling of the particle RNA, a rapidly labeled high molecular weight RNA and a long-lived, low molecular weight RNA were differentiated. Whereas the former was very sensitive to RNAase digestion, the latter was more resistant to degradation and was postulated to play a structural role in the arrangement of the proteins within the nRNP particles.

The long-lived low molecular weight RNA postulated by *Sekeris* and *Niessing* (1975) was detected recently by *Deimel* et al. (1977) and by *Northemann* et al. (1977).

When phenol extracts of 38 S nRNP particles or polyparticles – prepared in the presence of RNAase inhibitor – were analyzed by polyacrylamide gel electrophoresis in formamide, several low molecular weight RNA species could be identified (Fig. 4). 38 S nRNP particles showed 4 distinct RNA bands in the 5-8 S range and a zone of polydisperse low molecular weight material (a), whereas polyarticles exhibited at least 5 distinct RNA bands (b) in that range. The comparison of (a) and (b) showed that mono- and polyparticles have 4 RNA bands in common. From the fact that during the pre-

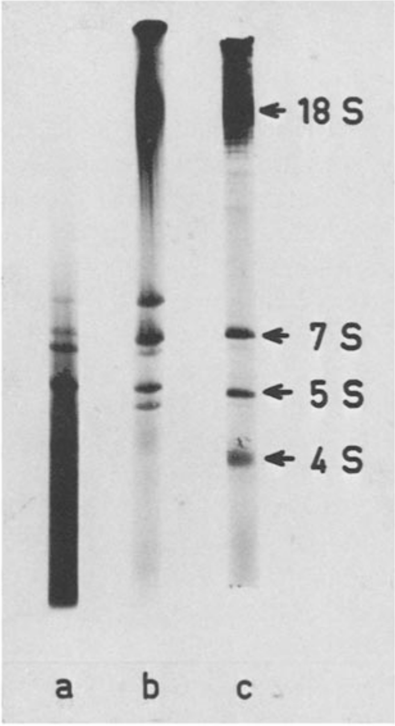


Fig. 4a-c. Polyacrylamide gel electrophoresis in formamide of RNA from mono- and polyparticles. Approximately 10-20 µg of RNA was applied to the gels. RNA extracted from polysomes of rabbit reticulocytes was used as standard. 38 S nRNP particles (a), polyparticles (b), polysomal RNA (c) (*Northemann* et al, 1977) paration of polyarticles RNA degradation is prevented by addition of cytosolic RNAase inhibitor, it may be concluded that the low molecular weight RNA species are not degradation products of hnRNA. Since 38 S particles show a similar small molecular weight RNA pattern as polyparticles, it can also be assumed that the 5-8 S low molecular weight RNA species of monoparticles are not cleavage products of hnRNA. Further evidence for the fact that the low molecular weight RNAs are no degradation products of hnRNA was shown by radioactivity measurements of the sliced polyacrylamide gels (Fig. 5). There was essentially no detectable radioactivity at the positions in the gel corresponding to the low molecular weight RNAs.

Raj et al. (1975) isolated nRNP particles from Novikoff hepatoma ascites cell nuclei by extraction with hypotonic buffer followed by Sepharose 6 B chromatography and sucrose gradient centrifugation. The particles with a buoyant density of 1.47 g/ml and a protein to RNA ratio of approximately 2 contained several low molecular weight RNA species with sedimentation constants in the range of 4 S - 8 S.

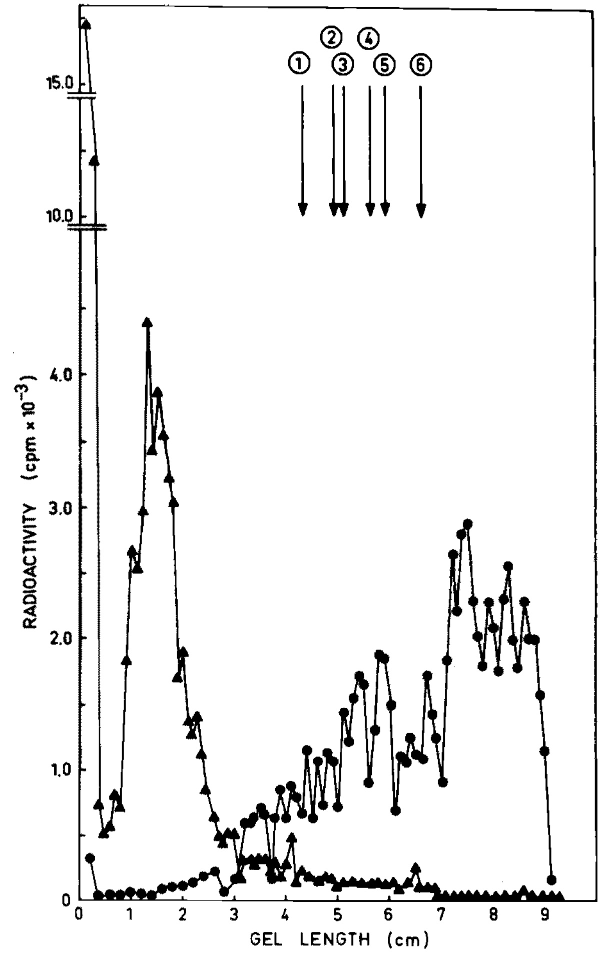


Fig. 5. Distribution of radioactivity after acrylamide gel electrophoresis of phenol extracted RNA from mono- and polyparticles. The gels shown in Fig. 4 were sliced and the radioactivity was determined. Monoparticles (●–●), polyparticles (▲–▲) The arrows above indicate the positions of the low molecular weight RNA species (*Scheurlen* et al, unpublished results)

Further evidence for the existence in nRNP particles of RNA regions which are resistant to RNAase digestion was presented by *Augenlicht* et al. (1976). Upon incubation of 76 S particles from HT 29 cells containing 15 S RNA with staphylococcal nuclease, an RNA-containing complex sedimenting at 2 S was isolated. The RNA component consisted of 26 nucleotides as estimated by formamide gel electrophoresis. Two protein species of 40,000 and 66,000 daltons were associated with this RNA. These proteins probably protect the RNA from digestion, since the protein-free RNA was completely digested by staphylococcal nuclease. *Stevenin* and *Jacob* (1974) isolated rat brain nRNP particles with a sedimentation coefficient of 75 S and a buoyant density of 1.39 g/ml. After incubation with a small amount of pancreatic RNAase, the density of these particles decreased to 1.35 g/ml, but was higher than that of free proteins (1.31 g/ml). Since the particles were labeled in both their RNA and protein moieties, it was possible to demonstrate an increase in the protein to RNA ratio after RNAase treatment. The nRNP complexes with a density of 1.35 g/ml seemed to contain RNAase-resistant RNA. Further studies on the digestion of nRNP particles from HeLa cells by RNAase have been carried out by *Kish* and *Pederson* (1975). After extensive digestion of nRNP particles with RNAases A and Tl, a low molecular weight complex remained. Fractionation by poly (U) Sepharose chromatography led to a poly (A)-rich ribonucleoprotein complex, containing two chraracteristic polypeptides of 74,000 and 86,000 daltons. Acrylamide gel electrophoresis of the RNA components revealed a poly (A) tract of 150-200 nucleotides and several oligo (A) segments of 20-30 nucleotides. Poly (A) sequences have been observed in 43 S particles isolated from Ehrlich ascites tumor cells (*Cornudella* et al., 1973) and in 30 S-60 S particles isolated from HeLa cells (*Ducamp* and *Jeanteur,* 1973).

From the work of *Molnar* and *Samarina* (1975) it is known that poly (A) sequences are complexed with specific proteins forming particles of 14 S, different from the 30 S-50 S monoparticles. Similar observations have been made by *Quinlan* et al. (1974), who were able to isolate from mouse ascites cells two poly (A) - containing structures of 15 S and 17 S, respectively. The 17 S particles were associated with approximately six proteins with molecular weights of 17,000-30,000 daltons, the 15 S particles with four proteins of higher molecular weight, particularly an 80,000 dalton species.

3.3. Properties of the Proteins

RNA and protein are the components of nRNP particles. Detailed studies on the protein part have been carried out. Great discrepancy concerning the number of different protein species involved in particle formation exists between different authors. *Samarina* et al. (1968), *Krichevskaya* and *Georgiev*

(1969), and *Lukanidin* et al. (1971) described only one polypeptide species with a molecular weight of 40,000 daltons, which they called "informatin." According to *Lukanidin* et al. (1972), 20-40 informatin molecules form aggregates, called "informofers." It was suggested that a monoparticle is a complex of one informofer and a short hnRNA chain. *Schweiger* and *Hannig* (1968), *Olsnes* (1970) and *Morel* et al. (1971) also found a small number of protein species (up to four polypeptides). On the other hand, many other groups observed a much larger number of proteins. *Niessing* and *Sekeris* (1971a) found 14, *Albrecht* and *van Zyl* (1973) 20, *Pederson* (1974a) 12-25, *Yoshida* and *Holoubek* (1976) 28, and *Gallinaro-Matringe* et al. (1975) 45 protein species (see also Table 2). The discrepancy between those workers who found only a small number of proteins and those who described a multitude of polypeptides cannot be due to the use of different species or tissues. *Niessing* and *Sekeris* (1971b) and *Gallinaro-Matringe* and *Jacob* (1974) could clearly demonstrate that the observed differences depended on the method used for the separation of the proteins. When the separation was carried out with a 6 *M* urea-acrylamide gel system at pH 4.5, only one major protein band was detected. Under these conditions, only positively charged proteins migrate, and separation occurs according to size and electric charge. On the other hand, when the proteins were separated according to their molecular weights in SDS polyacrylamide gels, a multitude of protein bands could be observed. The presence of only a single protein band observed after acid-urea-acrylamide gel electrophoresis may be partly due to the fact that some of the proteins, particularly phosphoproteins, do not enter the gels because of their acidic properties. Experiments of *Northemann* et al. (1978) showed that only one major protein band was found after polyacrylamide gel electrophoresis of nRNP particle proteins in 2.5 *M* urea at pH 2.7. When this "single" protein band was subjected to further SDS-acrylamide gel electrophoresis, it could be resolved into five distinct protein bands in the molecular weight range of 35,000-40,000 daltons.

Another possible explanation for the discrepancy in the number of protein species in nRNP particles observed by various authors could be due to the existence of different particle populations present in different ratios. *Kumar* and *Pederson* (1975) separated 76 S particles from HeLa cells into two classes by oligo (dT)-cellulose chromatography. Of the hnRNA, 10%-20% did not bind to oligo (dT)-cellulose and was associated with a single polypeptide of 40,000 daltons, while 80% was found in particles which displayed strong binding to oligo (dT)-cellulose and were characterized by a very complex population of proteins. However, the authors did not demonstrate that the material which did not bind to oligo (dT)-cellulose consisted of nRNP particles.

Figure 6 shows the typical protein pattern of rat liver nRNP particles obtained after SDS-acrylamide gel electrophoresis. A wide range of molecular weights was found for the nRNP particle proteins.

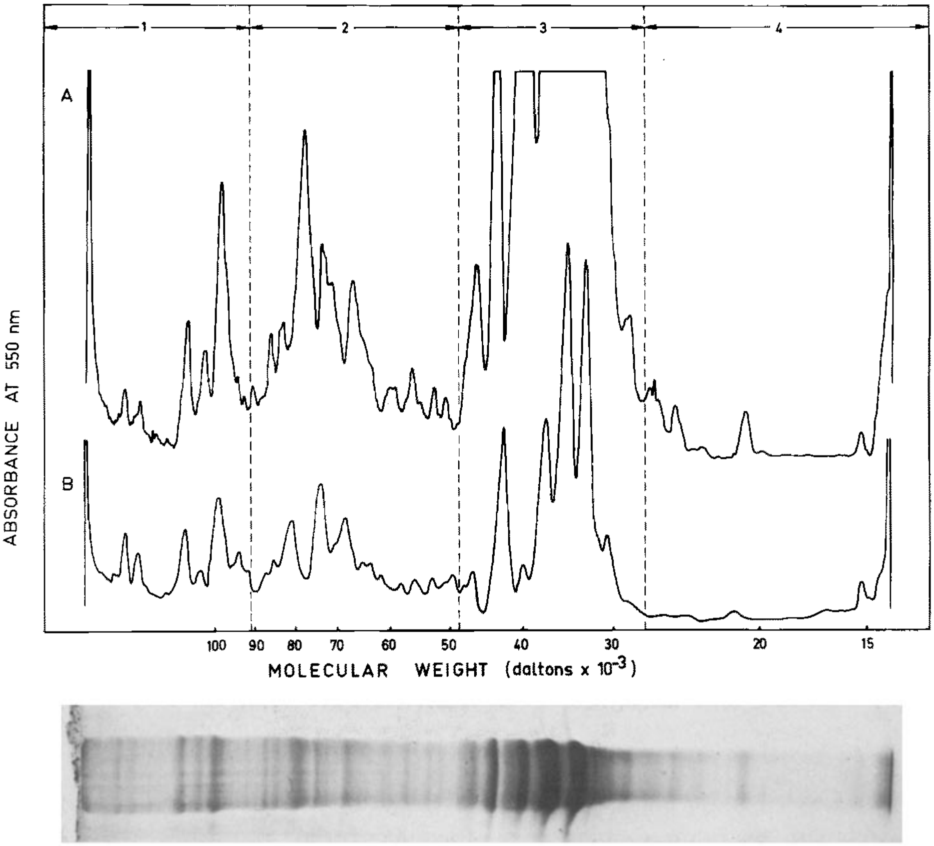


Fig. 6. SDS-acrylamide gel (10%) electrophoresis of rat liver 38 S nRNP particle proteins. (A) 40 μg, (B) 10 μg of particle proteins were applied to the gels. Densitometry was carried out at a wavelength of 550 nm

Polypeptide chains with molecular weights up to approximately 200,000 daltons were observed. Irrespective of the tissue or cell type used as starting material, many authors found the most prominent protein bands in the molecular weight range of 30,000-45,000 daltons. *Gallinaro-Matringe* et al. (1975) estimated these proteins to represent about 50% of the total protein. *Niessing* and *Sekeris* (1971a) as well as *Gallinaro-Matringe* and *Jacob* (1973) demonstrated that the protein patterns of polyparticles of different S values were the same.

While it is well-known that chromosomal proteins are modified in vivo by methylation, ADP-ribosylation, acetylation, and phosphorylation, only phosphorylation has been observed in nRNP particle proteins. *Schweiger* and *Schmidt* (1974) incubated 30 S particles in the presence of [γ-^{32}P] ATP. After sucrose gradient centrifugation a radioactive peak was found. The insolubility of the radioactive material in 10% trichloroacetic acid at 90°-95° C, its hydrolysis in 1.0 *N* NaOH at 100°C, and the identification of radioactivity in protein bands after polyacrylamide gel electrophoresis were regarded as evidence for the presence of phosphoproteins. Similar results were obtained by *Blanchard* et al. (1975). During incubation of nRNP particles from HeLa cell nuclei with [γ-^{32}P] ATP, radioactive phosphate was incorporated. *Stevenin* and *Jacob* (1974) injected [^{32} P] orthophosphate into rats and after a 4-h incorporation period nRNP particles were isolated from brain; 10% of the radioactivity found in the particles was RNAase resistant. Further treatment of the RNAase-resistant material with 10% trichloroacetic acid at 90°C left 25% of the initial radioactivity acid insoluble. The radioactivity in the protein fraction could be removed totally by pronase digestion, indicating the existence of phosphorylated proteins in the nRNP particles (*Gallinaro-Matringe* and *Jacob* 1973). After separation of nRNP particle proteins by SDS polyacrylamide gel electrophoresis, it was found that different protein species were phosphorylated, but a large amount of radioactivity was present mainly in two bands with electrophoretic mobilities corresponding to molecular weights between 30,000 and 40,000 daltons.

3.4. RNA-Protein Interactions

Nuclear sap exhibits a very complex protein pattern on SDS-polyacrylamide gel electrophoresis (*Stevenin* et al., 1975). The comparison with the protein pattern of nRNP particles reveals that nuclear sap and nRNP particles have several protein bands of identical electrophoretic mobility. It can be speculated that nuclear sap and nRNP particles have proteins in common, since during the formation of nRNP particles the newly synthesized RNA must be complexed with proteins which are then present in the nuclear sap. This is supported by the findings of *Zawislak* et al. (1974) who described in the case of rat brain the in vitro formation of an RNA-protein complex with properties similar to nRNP particles, when purified nuclear RNA and soluble nuclear sap proteins were incubated together. However, these artificial complexes were not as stable as native particles to NaCl treatment. Possibly an incorrect arrangement of RNA and proteins during the in vitro formation is responsible for this difference in stability. *Ishikawa* et al. (1974) also reacted isolated hnRNA with nuclear sap proteins from rat liver. They obtained complexes with sedimentation coefficients of less than 20 S. In spite of variation of protein to RNA ratios in the incubation mixtures, this value of 20 S could not be exceeded. This is in contrast to the findings of *Zawislak* et al. (1974) who obtained in vitro complexes of about 40 S. The discrepancy could be due to different RNA species used and different reconstitution conditions.

In one of the first studies published on nRNP particles from rat liver, *Samarina* et al. (1967b) dissociated and reconstituted 30 S particles. Dissociation was carried out in either 0.7 *M* KCl or 2.0 *M* NaCl. Sucrose gradient centrifugation of the dissociated particles revealed that no material sedimented at 30 S as shown by optical density measurements at 230 nm and determination of radioactive RNA. The authors concluded from these results that salt treatment dissociated the 30 S particles into RNA and protein. The protein components sedimented at 4 S - 6 S. The reconstitution of 30 S particles was achieved by the slow removal of the dissociating agents by dialysis. The reconstituted particles exhibited the same sedimentation properties, densities, and electron-microscopic appearance as the particles before dissociation. In contrast to these findings, the same group (*Lukanidin* et al., 1972) presented experimental evidence for the existence of RNA-free protein complexes with a sedimentation value of 30 S obtained from 30 S nRNP particles after treatment with 2.0 *M* NaCl. A more systematic study of the effect of NaCl on the RNA-protein interaction in nRNP particles has been carried out by *Stevenin* and *Jacob* (1974). Increasing NaCl concentrations of 0.4 *M*, 1.0 *M*, and 2.0 *M* led to a progressive release of 60, 75, and 80% of proteins from rat brain particles. The released material was present in a low molecular weight form of up to 15 S. RNA-free protein particles of 30 S were never detected. Some proteins remained bound to RNA, even after treatment with 2.0 *M* NaCl. It was found that phosphorylated proteins were more tightly bound to RNA in the presence of high concentrations of NaCl (*Gallinaro-Matringe* et al., 1975).

3.5. Models

Several models for the structure of nRNP particles have been proposed. The first model was presented by *Samarina* et al. (1968). They postulated the existence of 30 S protein particles, called informofers, composed of 20-40 identic polypeptides each of 40,000 daltons. According to their model, the hnRNA is localized on the surface of these protein complexes (Fig. 7a). This was concluded from the high sensitivity of the particle RNA to RNAases, indicating that the RNA was not protected against degradation by the protein of the particles. Furthermore, it was found by *Lukanidin* et al. (1972) that when hnRNA was removed from the 30 S particles by the use of high salt concentrations, 30 S RNA-free protein complexes were still conserved.

Observations contradictory to the model of *Samarina* et al. were made by *Stevenin* and *Jacob* (1974). Upon RNAase treatment of nRNP particles they found that the buoyant density decreased from 1.39 to 1.35 g/ml, a value between those of untreated monoparticles and of free proteins (1.31 g/ml). From this they concluded that a part of the particle RNA was protected against ribonuclease digestion by proteins. In addition, they could release proteins progressively from nRNP particles of a density of 1.39 g/ml

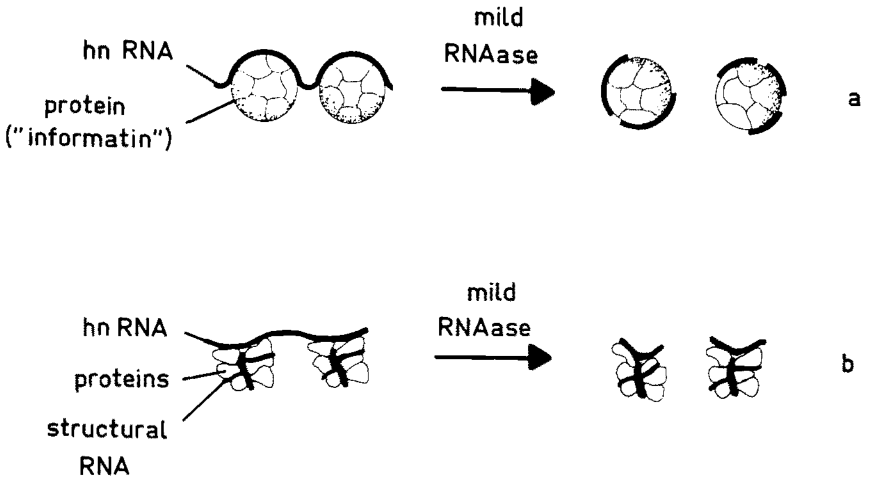


Fig. 7a and b. Models for the structure of nRNP particles. (a) model of *Samarina* et al., (1968), (b) model of *Sekeris* and *Niessing* (1975)

by treatment with increasing amounts of NaCl, and thus obtain nRNP particles with higher densities. They did not detect 30 S protein aggregates after dissociation with high salt. Since these results were not compatible with the informofer model of *Samarina* et al. (1968), *Stevenin* and *Jacob* (1972, 1974) proposed a "folded ribonucleoprotein strand" model. According to this model only a part of the RNA of nRNP particles is exposed and susceptible to RNAase degradation, whereas other regions of the RNA are protected by particle proteins. In addition, the proteins of the nRNP particles are very heterogeneous in size and cannot form stable aggregates of 30 S in the absence of RNA.

Sekeris and *Niessing* (1975) also demonstrated that a part of the RNA in nRNP particles was protected by protein against the action of RNAase. As already mentioned, they found an RNAase-sensitive, rapidly labeled high molecular weight RNA and in addition a stable, long-lived, low molecular weight RNA. These observations formed the basis of their model shown in Figure 7b. The low molecular weight RNA is located in the interior of the particles. It assembles with proteins and thus plays a structural role in the formation of monoparticles. The rapidly labeled high molecular weight hnRNA is attached to the surface of the monoparticles and links them together to form larger structures (polyparticles).

At the present time there are not sufficient experimental data to establish a generally accepted model for the structure of nRNP particles. However, it is very likely that the model proposed by *Samarina* et al. (1968) does not agree with the results obtained by many other groups.

3.6. Electron Microscopy

Electron microscopy has successfully been used to elucidate the ultrastructure of many subcellular components. Many authors have, therefore, examined nRNP particles by electron microscopy. *Samarina* et al. (1967a, 1968) provided evidence for the existence of a particulate structure of ribonucleoprotein complexes from rat liver nuclei. Different zones of a sucrose gradient containing nRNP particles were examined by electron microscopy using the shadowing technique. In the 30 S region, single spheric structures were visualized, while in the 45 S, 70 S-75 S, and 90 S-100 S zones predominantly dimers, trimers, tetra-to pentamers, and octa-to dodecamers were detected. The conversion of polyparticles into monoparticles could be achieved by mild RNAase treatment and visualized by electron microscopy (*Lukanidin* et al., 1972). After negative staining of monoparticles, a discoidal shape of 180 x 180 x 80 Å was inferred (*Samarina* et al., 1967a). *Monneron* and *Moulé* (1968) and *Albrecht* and *van Zyl* (1973) have studied monoparticles with diameters of 200-300 Å containing smaller subcomponents of 50-70 Å in diameter. A heterogeneous monoparticle population in rat brain has recently been described by *Stevenin* et al. (1976). After negative staining, different size classes with diameters of 100-300 Å were detected. When the particles were examined on ultrathin sections, they appeared smaller and had a fibrillar structure. The same structure was obtained when the ultrathin sections were treated by the regressive staining method of *Bernhard*, specific for ribonucleoprotein complexes (*Bernhard*, 1969). From these results it was suggested that the isolated particles and perichromatin fibrils are the same material.

4. Functional Aspects of Nuclear Ribonucleoprotein Particles

4.1. Relation to Subnuclear Structures

Thus far nRNP particles have been regarded as isolated nucleoplasmic organelles and only their structural aspects were discussed. To shed light on their physiologic function, their relation to the chromatin template as the site of RNA synthesis and to the nuclear membrane, the site of RNA translocation to the cytosol, has to be studied. Since nRNP particles contain rapidly labeled RNA, they may play an important role in the transfer of genetic information from the nucleus to the cytosol.

4.1.1. Relation to Chromatin

Tata and *Baker* (1974a, b) have reported that in rat liver nuclei the major part of the rapidly labeled RNA is associated with chromatin. After a short labeling period of 30 s with [^{3}H]uridine, *Augenlicht* and *Lipkin* (1976) found in HT 29 cells that no rapidly labeled RNA could be extracted in the form of nRNP particles. The total radioactivity was recovered in association with chromatin. After a labeling period of 90 or 120 s, 10 or 30% respectively of the rapidly labeled RNA could be extracted from chromatin. From these observations it was concluded that some of the chromatin-associated RNA was the precursor of nRNP-RNA. When chromatin was prepared from mouse myeloma S 194 cells with low salt, *Kimmel* et al. (1976) found that at least 80% of the nuclear RNA was bound to chromatin. The rapidly labeled RNA could be dissociated from chromatin under the same conditions as those employed for the preparation of nRNP particles from nuclei. When purified chromatin was incubated with exogeneous RNA or nRNP particles, no binding was observed, indicating that nRNP-chromatin complexes were not artifacts formed during the preparation of chromatin. This pointed to a functional association of the newly synthesized RNA with chromatin. The experiments of *Ishikawa* et al. (1974) showed that nRNP particles could be isolated from rat liver chromatin by the use of Mg^{2+}-chelating agents, such as EDTA, ATP, or sodium pyrophosphate. Since the newly synthesized RNA is present as an RNA-protein complex in the nucleoplasm, the question arises of when this complex formation occurs. In newt oocytes *Scott* and *Sommerville* (1974) showed that proteins from isolated nRNP particles were antigenically similar to nonbasic proteins of lampbrush loops. Antibodies were prepared against nRNP particle proteins. By means of immunofluorescence, the binding of these antibodies to different regions of the chromosomes of newt oocytes was studied. The antiserum reacted more intensively with the lampbrush loops – sites of active RNA synthesis – than with the chromomeres. In the Balbiani rings and other puffs of dipteran tissues it was found that proteins became associated with RNA while the RNA was still associated with chromatin (*Clever,* 1964; *Gall* and *Callan,* 1962). *Augenlicht* et al. (1976) incubated either chromatin or nRNP particles of HT 29 cells with staphylococcal nuclease. In both cases they isolated an RNAase-resistant fragment with a sedimentation coefficient of 2 S, complexed with the same proteins.

The existence of chromatin-nRNP particle complexes has also been observed in HeLa cells by *Bhorjee* and *Pederson* (1973). They prepared chromatin according to *Shaw* and *Huang* (1970) and analyzed the chromosomal proteins by SDS-gel electrophoresis. Several bands, particularly a 40,000 dalton protein, had the same electrophoretic mobility as proteins from isolated nRNP particles from HeLa cells. A method was therefore developed for the separation of nRNP complexes from chromatin. The crucial step of the preparation was the centrifugation of the chromatin-nRNP mixture through a sucrose cushion. In the resulting chromatin pellet, proteins characteristic for nRNP particles were greatly decreased. However, when this method was applied to the preparation of chromatin from rat brain (*Gattoni* et al., 1976), it failed to remove all the ribonucleoprotein particles. As a consequence *Gattoni* et al. (1976) have described an RNAase or NaCl treatment of chromatin before centrifugation through a sucrose cushion. By this step they succeeded in removing all nRNP particles, resulting in the loss of polypeptides characteristic for nRNP particles. Recently *Louis* and *Sekeris* (1976) have published a method for the isolation of nRNP particles from rat liver nuclei. After a short labeling period, EDTA-washed nuclei were extracted with an isotonic salt buffer under concomitant sonication. Using this method, *Northemann* et al. (1978) could isolate about 40% of the total nuclear radioactivity as RNA in nRNP particles. The balance of 60% of the radioactivity was found in chromatin. Additional amounts of 25% and 10% of nRNP particles could be dissociated from chromatin after two successive extractions with either isotonic or 0.22 and 0.3 *M* NaCl buffer solutions, respectively (Fig. 8).

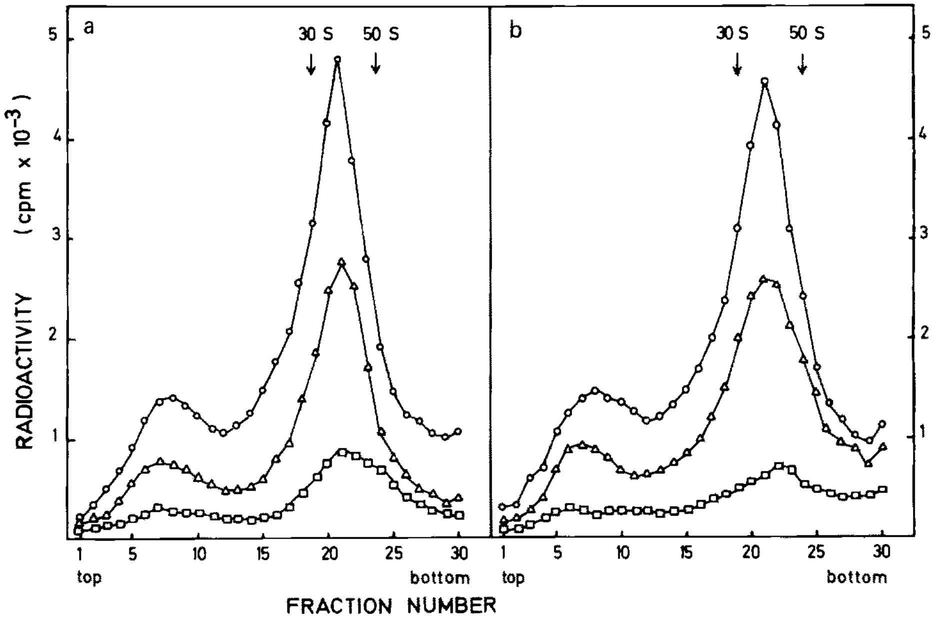


Fig. 8a and b. Sedimentation profiles of extracts of nuclei and chromatin in sucrose gradients. (a) Rat liver nuclei were extracted under sonication with salt (0.14 *M* NaCl)-buffer, pH 8.0. The chromatin obtained after centrifugation was subjected to two successive extractions with the same salt-buffer solution. All three extracts were layered on a 15 - 30% sucrose gradient and centrifuged. Acid precipitable radioactivity was determined in all fractions; first 0.14 M NaCl extract (○–○), second 0.14 M NaCl extract (△–△) and third 0.14 M NaCl extract (□–□). (b) Procedure as described under (a) with the exception that the chromatin was extracted with 0.22 *M* NaCl and further with 0.3 *M* NaCl in buffer, pH 8.0. Extracts of 0.14 *M* NaCl (○–○), 0.22 *M* NaCl (△–△), 0.3 *M* NaCl (□–□) (*Northemann* et al., 1978)

The close association of chromatin and ribonucleoprotein complexes has also been observed by electron microscopy. Perichromatin fibrils, first described by *Monneron* and *Bernhard* (1969) and *Fakan* and *Bernhard* (1971), are characterized by a diameter of 50-100 Å, close association with chromatin and high sensitivity to RNAase (*Bachellerie* et al., 1975). By autoradiographic experiments it could be demonstrated that perichromatin fibrils represented a morphologic state of newly formed hnRNA (*Petrov* and *Bernhard,* 1971; *Bachellerie* et al., 1975; *Fakan* et al., 1976). In addition to perichromatin fibrils, perichromatin granules of about 400 Å diameter were visualized (*Monneron* and *Bernhard,* 1969). The perichromatin granules seemed to be composed of irregularly coiled fibrils of about 30 Å thickness (*Puvion* and *Bernhard,* 1975). It was postulated that they represent a storage or transport form of newly synthesized hnRNA.

4.1.2. Relation to the Nuclear Membrane

Thus far, the association of nRNP particles with chromatin has been discussed. Much less information, however, is available on the mechanism of translocation of the nRNP particles from chromatin to the cytosol. An interesting hypothesis concerning a connection between chromatin and cytoplasm was provided recently by *Faiferman* and *Pogo* (1975). Nuclei were disrupted by methods avoiding shearing forces, for example by treatment with high salt (0.8 *M* NaCl)-buffer-DNAase or by use of low pressure in a nitrogen cavitation bomb. Under these conditions they isolated a fibrogranular material associated with nuclear membranes which they called "RNP network." The membrane-bound RNP network consisted of 63% protein, 14% RNA, 0.4% DNA, and 22.6% lipids. Treatment with triton X-100 decreased the lipids to 2.2% and removed the nuclear envelope, which could be visualized electronmicroscopically. RNAase treatment, on the other hand, resulted in the loss of main parts of the RNP network, whereas the membrane structures were conserved. Digestion with pronase prior to the RNAase action led to the complete disappearance of the fibrogranular material.

4.2. Evidence for the Existence of Message in the Particles

It is widely accepted that hnRNA is a precursor for cytoplasmic mRNA. The experimental evidence for a precursor-product relationship of hnRNA and mRNA is based on kinetic data (*Georgiev,* 1967; *Jelinek* et al., 1973; *Scherrer* and *Marcaud,* 1968; *Perry* et al., 1974; *Puckett* et al., 1975), hybridization studies (*Macnaughton* et al., 1974; *Imaizumi* et al., 1973; *Herman* et al., 1976; *Ross* et al., 1976; *Curtis* and *Weissmann,* 1976), experiments with virus-transformed cells (*Lindberg* and *Darnell,* 1970; *Wall* and *Darnell,* 1971; *Wall* et al., 1973), and in vitro translation of hnRNA (*Schutz* et al., 1972; *Ruiz-Carrillo* et al., 1973; *Knöchel* and *Tiedemann,* 1975).

In order to demonstrate that the rapidly labeled RNA found in the nRNP particles plays a role as precursor for mRNA, experiments similar to those mentioned above would be desirable. Initial experiments along this line have been carried out with 30 S nRNP particles from mouse ascites cells by *Kinniburgh* and *Martin* (1976a, b). They isolated poly (A) -containing mRNA from polysomes and synthesized the complementary DNA (cDNA). From 30 S nRNP particles an RNA < 4 S was isolated and hybridized with the cDNA. Compared to the hybridization rate of the homologous system (poly (A) + mRNA against cDNA), the rate for the reaction of cDNA with the RNA from the particles was found to be 100 times slower. When the 30 S particle RNA was further purified, it reacted faster with the (poly (A) + mRNA) -specific cDNA than crude particle RNA. From the hybridization kinetics *Kinniburgh* and *Martin* (1976a) concluded that at least 85% of the cytoplasmic poly (A) + mRNA had a counterpart in the particle RNA. In addition, it was estimated that 10 - 15% of the particle RNA was precursor for cytosolic poly (A) + mRNA.

4.3. Function of Particle Proteins

The major part of the nRNP particles consists of protein. Nevertheless, except for a few examples discussed below, only speculations as to their function can be made.

From the experiments in which nRNP particles were treated with RNAases, it can be assumed that one of the roles of the protein may be the stabilization of newly synthesized RNA. It may be that the limited degradation of hnRNA during processing occurs at RNA sequences which are not specifically protected by protein. Thus far, a correlation of specific proteins with certain RNA sequences has only been shown in the case of poly (A).

Specific poly (A) -binding proteins were first described in ribonucleoprotein complexes in the cytosol. Two proteins with molecular weights of approximately 50,000 and 75,000 daltons were observed in these complexes (*Morel* et al., 1971; *Kwan* and *Brawerman,* 1972; *Kumar* and *Lindberg,* 1972; *Blobel,* 1972 and 1973; *Bryan* and *Hayashi,* 1973; *Lindberg* and *Sundquist,* 1974; *Lebleu* et al., 1971; *Gander* et al., 1973; *Irwin* et al., 1975; *Barrieux* et al., 1976; *Kish* and *Pederson,* 1975; *Schwartz* and *Darnell,* 1976; *Schweiger* and *Mazur,* 1976). The existence of poly (A) -binding proteins with molecular weights of 74,000 and 86,000 daltons in nRNP particles was demonstrated by *Kish* and *Pederson* (1975). The authors assumed that the major poly (A) -binding protein of 74,000 daltons was probably bound both to the nuclear and to the cytoplasmic poly (A) sequences. It might therefore play a role in the translocation of message from the nucleus to the cytosol.

For the various posttranscriptional modification reactions many enzymes are necessary. Whether these enzymes are components of the nRNP particles or part of the nuclear sap is not known.

RNAases play a key role in the processing of hnRNA. *Niessing* and *Sekeris* (1970) incubated the proteins from 30 S nRNP particles with isolated high molecular weight hnRNA from rat liver and observed a limited degradation of the hnRNA, suggesting the presence of an endonuclease in the 30 S nRNP particles. Experiments of *Scheurlen* (unpublished results) have shown that at least two different RNAase activities, assayed with poly (A) and high molecular weight yeast RNA, are present in the nuclear sap. Both enzymes show sedimentation properties in a sucrose gradient different from those of nRNP particles (Fig. 3). They seem, therefore, not to be included among the particle proteins.

Essential enzymes in the posttranscriptional modifications are the homopolymer synthetases. *Niessing* and *Sekeris* (1972, 1973, and 1974) were able to identify enzymes in rat liver 30 S nRNP particles which catalyzed the formation of ribohomopolymers. *Louis* and *Sekeris* (1977) have recently studied the subnuclear distribution of these enzymes in rat liver. The various enzymes could be extracted from nuclei to different degrees with 0.14 and 0.3 *M* NaCl – conditions used for the isolation of nRNP particles. The poly (U) polymerase was most easily extractable, followed by a Mn^{2+}-dependent poly (A) polymerase. The Mg^{2+}-dependent poly (A) polymerase was most tightly bound to chromatin. A part of the extracted enzymes was bound to nRNP particles as shown by sucrose gradient centrifugation.

Kish and *Kleinsmith* (1974) have demonstrated the existence of 12 distinct protein kinases in the nonhistone protein fraction of beef liver chromatin. Phosphoproteins are known to be present in chromatin and in nRNP particles. Enzymes associated with nRNP particles and capable of phosphorylating particle proteins have been described by *Schweiger* and *Schmidt* (1974) and by *Blanchard* et al. (1975). It is not known whether any of the protein kinases which have been isolated from chromatin and the enzyme(s) associated with nRNP particles are identical.

5. Alterations in Nuclear Ribonucleoprotein Particles

One possible approach to learning more about the function of nRNP particles consists in the use of hormones or drugs which interfere with the mechanism(s) of gene expression.

Pederson (1974b) studied the effect of hydrocortisone on rat liver nRNP particles. Hydrocortisone stimulated the synthesis of hnRNA, as measured by the incorporation of [^{3}H]orotate. Concomitantly, an increased synthesis of nRNP particle proteins measured by the incorporation of tritiated amino acids was observed. There was no detectable effect on the chromosomal proteins when these were well-separated from nRNP particles. From these findings, *Pederson* concluded that the increased synthesis of nonhistone proteins after stimulation of gene activities with steroid hormones might reflect a contamination of chromatin with nRNP particles.

Knowler (1976) measured RNA synthesis in isolated rat uteri. After administration of estradiol, rat uteri were dissected and incubated in vitro with [^{3}H]uridine. An up to eight-fold increase could be demonstrated in [^{3}H]uridine incorporation into the RNA of nRNP particles, which were isolated according to the diffusion method of *Samarina* et al. (1968).

Nuclear RNP particles were examined also during the process of chemical carcinogenesis. It was found by *Yoshida* and *Holoubek* (1976) that the carcinogenic 3'-methyl-4-diethylaminoazobenzene had a high affinity for the 30 S nRNP particle proteins from rat liver. In addition, alterations in the protein moiety of the nRNP particles were observed by *Patel* and *Holoubek* (1976). After 10 weeks of feeding of the carcinogenic 3'methyl-4-diethylaminoazobenzene, a two-dimensional polyacrylamide gel electrophoresis showed the absence of one of the major particle proteins. This effect was not obtained when the noncarcinogenic 4-aminoazobenzene was used as control.

The effects of α-amanitin and actinomycin D on nRNP particles from rat liver were recently studied by *Louis* and *Sekeris* (1976). They observed a reduction of 20-40% in the yield of nRNP particle proteins 2 hours after the administration of these drugs. The incorporation of radioactively labeled orotate into hnRNA decreased to 60 - 85% of the controls after α-amanitin and actinomycin D, and the buoyant density of nRNP particles measured in CsCl gradients increased slightly. A relative depletion of the protein component in the particles was suggested.

Gross et al. (1977) compared the effects of D-galactosamine and actinomycin D on nRNP particles of 38 S from rat liver. Nuclear RNA synthesis as measured by the incorporation of [^{3}H]cytidine was reduced to approximately 20% of controls 3 hours after the administration of galactosamine or actinomycin D, respectively. Although the synthesis of RNA was inhibited to the same extent by both treatments, the yield of nRNP particles dropped to 41% of controls after galactosamine treatment, but only to 78% after actinomycin D administration. It is not yet clear whether this difference is caused by an increased loss of nRNP particles after galactosamine treatment either due to a stimulated transport or to a leaky nuclear envelope, or by the inhibition of the nucleocytoplasmic translocation of nRNP particles after actinomycin D.

6. Conclusion

The mechanisms of mRNA formation in eukaryotes differ radically from those in prokaryotes. From the number of steps involved in the mRNA manufacture, it is clear that many sites for the control of transcription may exist. The primary transcript of chromatin, hnRNA, is modified by polyadenylation, cap formation, and methylation, processed in endo- and exonucleolytic reactions and translocated to the cytosol. During all these steps, the RNA can never be detected in a naked form, being always associated with proteins. All the events mentioned therefore occur on RNA as part of a complex with proteins. Most studies on the mechanisms of transcription in eukaryotes have been carried out with nuclear RNA irrespective of the involved proteins. However, a full understanding of the mRNA formation including the transport to the cytosol will require an appreciation of the structure and function of the nuclear ribonucleoprotein complexes. Nuclear RNA-protein complexes can be isolated in form of mono- or polyparticles from nuclei. They contain rapidly labeled RNA assumed to be hnRNA. Although there is convincing evidence for mRNA sequences in hnRNA, only a few indications for the existence of message in the RNA of nRNP particles are available. Further investigations are necessary to establish that the particle RNA contains pre-mRNA.

Many questions concerning the role of the proteins in the nRNP particles remain to be answered. How do the proteins influence the three-dimensional structure of the particles? How do the proteins protect the hnRNA against the action of nuclear RNAases? Does the binding of proteins to hnRNA result in the exposure and a higher susceptibility of hnRNA regions to RNAases and other enzymes involved in processing? Which of the particle proteins are enzymes? What role do the proteins associated with the nRNP particles play during the translocation of message from the nucleus to the cytoplasm?

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Action and Uptake of Neurotransmitters in CNS Tissue Culture

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Contents

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1. Introduction

In 1907 *Harrison* was able for the first time to cultivate frog nervous tissue in vitro. Since that time, nervous tissue of many different species has been cultivated and a variety of culture techniques have been developed (for refs. see *Murray,* 1965, 1971; *Herschman,* 1974; *Nelson,* 1975b; *Varon,* 1975). The different types of cultures, ranging from organotypic or explant cultures to dissociated cells and clonal cell lines, and their usefulness in studying specific research problems have been described extensively (*Murray,* 1971; *Herschman,* 1974; *Nelson,* 1975b; *Varon,* 1975; *Crain,* 1976). The present review is mainly concerned with studies performed in organotypic cultures of the central nervous system (CNS), although investigations on dissociated cells and cell lines and some results on cultures of the peripheral nervous system have also been included.

It has been reported by many laboratories that nervous tissue cultivated in vitro maintains and develops morphologic and functional properties similar to nervous tissue in situ (*Murray,* 1965, 1971; *Crain,* 1966, 1975, 1976; *Varon,* 1975). The tissue culture technique offers an excellent opportunity to record bioelectric activities from neurones and glial cells with microelectrodes under direct microscopic observation. This allows the identification of the various cell types by means of morphologic criteria and the correlation of electrophysiologic and morphologic features (*Hild* and *Tasaki,* 1962; *Crain,* 1966; *Hösli* et al., 1973c, 1975b, 1976a; *Nelson,* 1975b; *Ransom* and *Nelson,* 1975; *Varon,* 1975). It also provides a valuable tool for studying the effects of neurotransmitters, most of which do not pass the blood-brain barrier, on neurones and glial cells of the mammalian CNS, and to investigate ionic mechanisms associated with transmitter actions by altering the composition of the extracellular environment (*Hösli* et al., 1973a, c; 1975b, 1976a; *Ransom* and *Nelson,* 1975).

Another interesting research field using this method is the investigation of the cellular and fine-structural localization of the uptake of neurotransmitters in CNS tissue using autoradiographic techniques. Light-microscopic and electron-microscopic studies have shown that cells in tissue culture are usually better preserved than in slices or homogenates of CNS tissue (*Hökfelt* and *Ljungdahl,* 1972a, b; *Hösli* et al., 1972a), in which most of the uptake studies have been performed.

The present review is mainly concerned with electrophysiologic studies of the action of neurotransmitters and with autoradiographic investigations of the uptake of transmitter substances in tissue cultures of the mammalian CNS. The results obtained are compared with observations made in the CNS in situ.

2. Cytologic and Histochemical Properties of Neurones and Glial Cells in Tissue Culture

2.1. Outgrowth Pattern and Cytologic Properties

Many studies have shown that nervous tissue grown in culture maintains and/or develops morphologic properties similar to nervous tissue in situ. Extensive reviews on the ontogenetic development and maintenance of organotypic and dissociated cell cultures have been written by *Murray* (1965, 1971) and *Crain* (1966), and more recently by *Herschman* (1974), *Nelson* (1975 a, b), *Varon* (1975), *Varon* and *Saier* (1975), and *Mandel* et al. (1976). Using light-microscopic and electron-microscopic techniques, it has been shown that neurones and glial cells mature and differentiate in culture and that new synaptic complexes are formed. Furthermore, myelination of explant cultures usually occurs after approximately 2-3 weeks in vitro. The pattern of outgrowth, which varies considerably between cultures of different regional origin, and the cytologic properties of the various cell types of CNS cultures have been described in detail by many authors (*Okamoto*, 1958; *Bunge* et al., 1965; *Murray*, 1965, 1971; *Peterson* et al., 1965; *Guillery* et al., 1968; *Sobkovicz* et al., 1968; *Kim*, 1970, 1976; *Seil* and *Herndon*, 1970; *Hösli* et al., 1973c, 1974, 1975b; *Privat* et al., 1973). The differences in cytologic features and outgrowth pattern were also dependent on the ontogenetic development of the nervous tissue at the time of explantation. Thus, in organotypic cultures of spinal cord and brain stem of human fetuses (8-18 weeks in utero) and of fetal rats (16-18 days in utero), there was usually a greater number of mature and differentiated cells than in cultures prepared from cerebral cortex or cerebellum (*Hogue*, 1947; *Peterson* et al., 1965; *Hösli* et al., 1973 d). After 2-3 weeks in vitro, however, neurones showing morphologic features of cortical pyramidal cells or cerebellar Purkinje cells, respectively, could also be frequently observed (*Hogue*, 1947; *Lapham* and *Markesbery*, 1971; *Hösli* et al., 1973d; *Markesbery* and *Lapham*, 1974; *Hauw* and *Escourolle*, 1975).

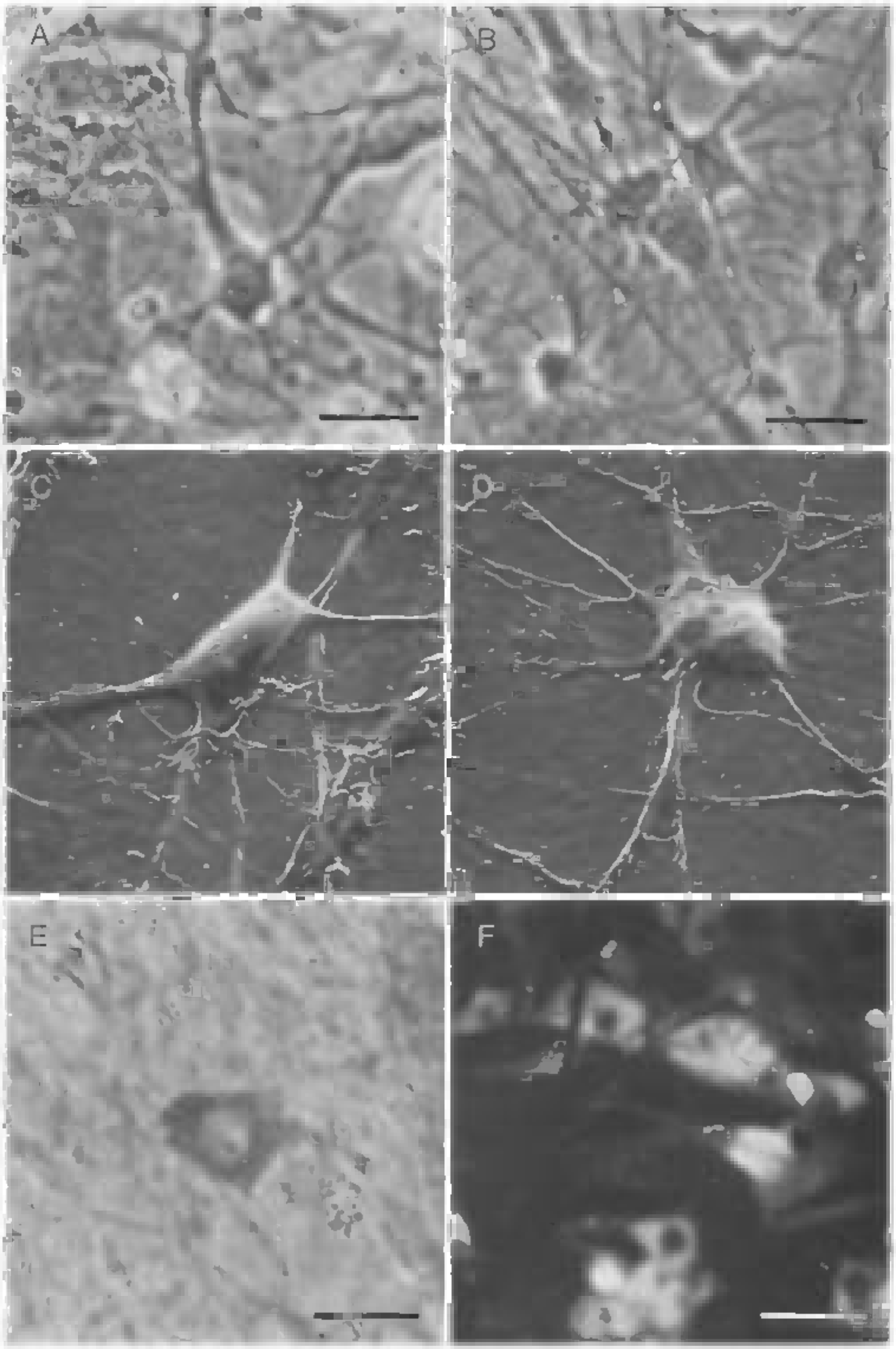
Neurones in tissue culture are usually identified by their size, shape, and nuclear morphology as well as their location in the cultures (*Hogue*, 1947; *Bornstein* and *Murray*, 1958; *Okamoto*, 1958; *Murray*, 1965; *Peterson* et al., 1965; *Hösli* et al., 1973d, 1974, 1975b). The nuclear membrane of the neurones is usually well defined and the nucleus contains one prominent nucleolus (Fig. 1A). Several large dendrites arise from the cell body, branching into thinner dendrites (Fig. 1A, C). Axons which are usually thinner than dendrites can only rarely be identified. In contrast to glial cells, the majority of neurones remain in or at the edge of the explant, being surrounded by astrocytes and their processes, and only a small number of neurones migrate or are passively pulled by other cells into the outgrowth zones (*Murray,* 1965; *Peterson* et al., 1965; *Sobkovicz* et al., 1968; *Hösli* et al., 1973d, 1974, 1975b; *Varon,* 1975). In electron-microscopic studies, the nuclear membrane of neurones shows prominent folds and indentations. The cytoplasm contains one to several Golgi complexes, small elongated mitochondria, and many ribosomes (*Bunge* et al., 1965; *Sobkovicz* et al., 1968; *Hösli* et al., 1975b).

The cell bodies of the astrocytes vary considerably in size and shape. The relatively large nucleus appears more or less oval and usually contains two or more small intranuclear densities (Fig. 1B). A great number of glial cells migrate into the outgrowth zones of the cultures. The numerous processes arising from the cell body of glial cells (Fig. 1D) often form a dense network together with the outgrowing neurites (Fig. 1B). Electron-microscopic studies reveal that the nuclear membrane of glial cells is less frequently and less deeply indented than that of neurones. The cytoplasm contains rough endoplasmic reticulum, free ribosomes, and cytoplasmic filaments with a diameter of 6-12 μm (*Guillery* et al., 1968; *Hösli* et al., 1975b). The perikarya and processes of most glial cells are separated by an intracellular cleft of 10-40 nm. Typical gap junctions were only found between glial cells located close to the explant but not in the outgrowth zones (*Guillery* et al., 1970; *Wolff* et al., 1971).

Basement membranes (basal lamina) have been observed at the basal layer of the explant and between neural elements and newly formed collagen from meningeal or vascular sources (*Guillery* et al., 1970; *Wolff* et al., 1974) as well as between migrating glial cells and the collagen substrate of the culture (*Wolff* et al., 1971).

Cells identified as oligodendrocytes frequently occur in cultures of cerebellum, cortex, and brain stem, whereas in spinal cord cultures only very few oligodendrocytes could be observed. Oligodendrocyte-like cells were often seen in the early phase of the outgrowth (*Hogue,* 1947; *Hösli* and *Hösli,* 1971; *Wolff* et al., 1971; *Hösli* et al., 1975b). However, most of these cells degenerated a few days after migration.

Fig. 1. (A) Phase contrast picture of a multipolar neurone in the outgrowth zone of a ▶ brain stem culture (human fetus, 14 weeks in utero, 8 days in vitro). *Bar:* 20 μm (*Hösli* et al., 1975b). (B) Astrocytic network in the zone of migration of a 22-day-old rat spinal cord culture. Phase contrast. *Bar:* 30 μm (*Wolff* et al., 1971). (C, D) Scanning electron-microscopic pictures, magnification: × 1900 (C) Neurone in the outgrowth zone of a human spinal cord culture (10 days in vitro, fetus 18 weeks in utero). (D) Astrocyte lying in the outgrowth zone of a human spinal cord culture (10 days in vitro; fetus 18 weeks in utero) (*Hösli* et al., 1974). (E) Spinal neurone of a culture (15 days in vitro) from a human fetus of 12 weeks in utero showing a high AChE content. *Bar:* 20 μm (*Hösli* et al., 1974). (F) Fluorescence microscopic picture of monoamine-containing neurones. Rat brain stem culture, 14 days in vitro. *Bar:* 30 μm (*Hösli* et al., 1973e)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The development of synaptic contacts in maturing CNS cultures has been described extensively by several authors (*Bunge* et al., 1965, 1967; *Murray,* 1965; *Grainger* et al., 1968; *Guillery* et al., 1968; *Bird* and *James,* 1973; *Tunnicliff* and *Kim,* 1973; *Privat* et al., 1974; *Kim,* 1976). Synaptic junctions, mainly axodendritic and axosomatic synapses, frequently occur in the dense zones (explant and its margin) of the cultures, whereas neurones in the outgrowth zones have either only very few synaptic contacts or none (*Bunge* et al., 1965, 1967; *Guillery* et al., 1968; *Hösli* et al., 1975b)

2.2. Histochemical Properties

Information on enzymic maturation of cultured neurones and glial cells has been obtained by many laboratories using biochemical and histochemical techniques (*Hösli* and *Hösli,* 1970, 1971; *Lehrer* et al., 1970; *Murray,* 1971; *Seeds,* 1971; *Kim* et al., 1972, 1974, 1975; *Rosenberg,* 1972; *Herschman,* 1973, 1974; *Peterson* et al., 1973; *Hösli* et al., 1975c; *Mandel* et al., 1976; *Honegger* and *Richelson,* 1976). It has been reported that cultured neurones are able to store and/or to synthesize various enzymes which are known to be present in the nervous system in situ (*Murray,* 1971; *Herschman,* 1973, 1974; *Hösli* et al., 1975c; *Mandel* et al., 1976).

2.2.1. The Presence and Ontogenetic Development of Acetylcholinesterase (AChE)

From histochemical studies in the CNS in situ, it is well known that there is a considerable number of neurones with a high AChE content in the spinal cord and brain stem, whereas in the cerebellum there is only a small number of AChE-containing cells (*Giacobini,* 1959; *Koelle,* 1963; *Silver,* 1967). It has been reported that Purkinje cells of adult rat do not contain AChE, whereas a large amount of AChE is found in Purkinje cells during the first 3 weeks of postnatal development (*Csillik* et al., 1964). In organotypic cultures of rat spinal cord, groups of large neurones with a high AChE content (probably motoneurones) have been observed (*Hösli* and *Hösli,* 1971; *Kim* et al., 1972, 1975; *Tischner* and *Thomas,* 1973; *Hösli* et al., 1975c). In brain stem cultures of the rat, a relatively great number of large neurones with a high AChE activity could also be demonstrated (*Hösli* and *Hösli,* 1970; *Hösli* et al., 1975c), being similar to the intensely stained neurones of the hypoglossal nucleus and of the nucleus ambiguus observed in brain stem sections of newborn rats (*Wolf* et al., 1975). AChE acitivity was also observed in Purkinje cells of rat cerebellar cultures up to 20 days in vitro (*Kim* and *Murray,* 1969; *Hösli* and *Hösli,* 1970; *Ieradi* and *Cataldi,* 1972; *Minelli* et al., 1971; *Hösli* et al., 1975c). This result is in agreement with observations made by

Csillik et al. (1964) in the rat cerebellum in situ, who also described a high AChE activity in Purkinje cells up to 3 weeks after birth. AChE-containing neurones were also seen in cultures of the optic tectum of chick embryos (*Minelli* et al., 1971), in cultured sympathetic ganglia (*Hermetet* et al., 1970; *Kim* and *Munkacsi,* 1972; *Hervonen* and *Rechardt,* 1974; *Perry* et al., 1975), in spinal ganglia (*Ciesielski-Treska* et al., 1970; *Minelli* et al., 1971), and in cultured retina (*Hansson,* 1966). Furthermore, neurones in dissociated cultures (*Sensenbrenner* et al., 1972; *Contestabile* et al., 1973; *Kim* and *Wenger,* 1973; *Peterson* et al., 1973; *Mandel* et al., 1976) and cultured neuroblastoma cells (*Blüme* et al., 1970; *Amano* et al., 1972) also stained for AChE. In contrast to neurones, glial cells did not contain AChE but butyrylcholinesterase (*Kim* and *Murray,* 1969; *Hösli* and *Hösli,* 1970, 1971; *Hösli* et al., 1975c).

Biochemical and histochemical investigations on the ontogenetic development of AChE in CNS cultures of various species have shown an increase of this enzyme during maturation in vitro (*Geiger* and *Stone,* 1962; *Yonezawa* et al., 1962; *Minelli* et al., 1971; *Werner* et al., 1971; *Ieradi* et al., 1972; *Kim* et al., 1972, 1974, 1975; *Sensenbrenner* et al., 1972; *Contestabile* et al., 1973; *Tischner* and *Thomas,* 1973; *Hösli* et al., 1975c; *Honegger* and *Richelson,* 1976). Studies of the presence of AChE in cultures of fetal human spinal cord at different stages of development have demonstrated that cultured neurones of human fetuses of 12-18 weeks in utero have a considerable higher AChE content than neurones of fetuses of 7-9 weeks (*Hösli* et al., 1974, 1975c). Figure 1E illustrates a cultured human spinal neurone from a 12-week-old fetus with a high AChE activity in the soma and dendrites (*Hösli* et al., 1974). A similar increase of the AChE activity during ontogenetic development has also been described in sections of fetal human and rat spinal cord and brain stem (*Duckett* and *Pearse,* 1969; *Sarrat,* 1970; *Wolf* et al., 1975).

2.2.2. Other Enzymes

Biochemical and histochemical studies demonstrating the presence of various other enzymes, e.g., choline acetyltransferase, monoamine oxidase, lactate dehydrogenase, succinic dehydrogenase, glutamate dehydrogenase, and alkaline and acid phosphatase in cultured nervous tissue have been reported by many laboratories (*Yonezawa* et al., 1962; *Hermetet* et al., 1970; *Lehrer* et al., 1970; *Minelli* et al., 1971; *Murray,* 1971; *Seeds,* 1971; *Kim* et al., 1972, 1974, 1975; *Rosenberg,* 1972; *Herschman,* 1973, 1974; *Kim* and *Wenger,* 1973; *Hösli* et al., 1975c; *Honegger* and *Richelson,* 1976). The pattern of development of these enzymes was also found to evolve parallelly with the maturing of the cultured nervous tissue (*Lehrer* et al., 1970; *Minelli* et al., 1971; *Seeds,* 1971; *Kim* et al., 1972, 1974, 1975; *Rosenberg,* 1972; *Sensenbrenner* et al., 1972; *Herschman,* 1973; *Hösli* et al., 1975c; *Honegger* and *Richelson,* 1976, *Mandel* et al., 1976), indicating that the technique of tissue culture provides a valuable tool for the examination of morphologic and histochemical maturation and differentiation of the developing nervous system.

2.2.3. Monoamines

The occurrence of monoamine transmitters in nervous tissue cultures using the technique of fluorescence microscopy described by *Falck* et al. (1962) was demonstrated in cultured sympathetic ganglia (*Sano* et al., 1967; *Hermetet* et al., 1968; *Eränkö* et al., 1972; *Kim* and *Munkacsi*, 1972; *Benitez* et al., 1973; *Mains* and *Patterson*, 1973; *Hervonen*, 1974, 1975; *Jacobowitz* and *Greene*, 1974; *Perry* et al., 1975; *Webb* et al., 1975) and in cultures of hypothalamus (*Benitez* et al., 1968) and rat brain stem (Fig. 1F, *Hösli* et al., 1971a, 1973e). Monoamine-containing neurones have previously been found in sympathetic ganglia (*Norberg* and *Hamberger*, 1964; *Blümcke* and *Niedorf*, 1965; *Iversen*, 1967; *Olson* and *Malmfors*, 1970) and in various regions of the CNS in situ including the hypothalamus (*Lichtensteiger* and *Langemann*, 1966; *Lichtensteiger*, 1969) and the brain stem (*Carlsson* et al., 1962; *Dahlström* and *Fuxe*, 1964, 1965), indicating that cultured neurones retain histochemical properties similar to those studied in vivo.

3. Autoradiographic Localization of the Uptake of Neurotransmitters in CNS Tissue Culture

There is considerable evidence that specific uptake mechanisms are involved in terminating the action of certain transmitter substances such as amino acids and monamines (*Iversen*, 1967; *Iversen* and *Neal*, 1968; *Snyder* et al., 1970; *Neal*, 1971; *DeFeudis*, 1975). Studies on uptake kinetics, which have mainly been carried out on slices or synaptosomes of CNS tissue, have demonstrated that most amino acid transmitters, e.g., GABA, glycine, glutamate, and aspartate, are taken up by high-affinity transport systems (*Johnson* and *Aprison*, 1970; *Snyder* et al., 1970; *Iversen* and *Johnston*, 1971; *Johnston* and *Iversen*, 1971; *Wofsey* et al., 1971; *Arregui* et al., 1972; *Logan* and *Snyder*, 1972; *Balcar* and *Johnston*, 1973; *Levi* and *Raiteri*, 1973). Investigations on uptake kinetics of amino acids in nervous tissue grown in culture have shown that cultured retina, cerebrum, cerebellum, and spinal cord also possess a high-affinity uptake system for GABA, glycine, and glutamate (*Cho* et al., 1973, 1974; *Tunnicliff* et al., 1973, 1974, 1975; *Lasher*, 1975; *Tunnicliff*, 1975). There is also evidence from autoradiographic and biochemical studies that glial cells are involved to a great extent in the uptake of amino acid transmitters (*Faeder* and

Salpeter, 1970; *Ehinger* and *Falck,* 1971; *Henn* and *Hamberger,* 1971; *Hökfelt* and *Ljungdahl,* 1971b; *Orkand* and *Kravitz,* 1971; *Ehinger,* 1972; *Hösli* and *Hösli,* 1972, 1976a, b; *Hösli* et al., 1972a, 1973c, 1975b; *Henn* et al., 1974; *Schon* and *Iversen,* 1974; *Schon* and *Kelly,* 1974a, b) and that glial uptake exhibits characteristics of a high-affinity transport system closely resembling that into nerve terminals (*Henn* and *Hamberger,* 1971; *Faivre-Baumann* et al., 1974; *Hutchison* et al., 1974; *Schrier* and *Thompson,* 1974; *Iversen* and *Kelly,* 1975; *Schubert,* 1975; *Sellström* and *Hamberger,* 1975).

The method of tissue culture is a valuable tool for studying the uptake of transmitter substances using autoradiography for the following reasons: In contrast to investigations in slices or after intraventricular or intracerebral injections in situ, the diffusion of the isotopes in the cultures is very fast, allowing relatively short incubation times (30 s to a few minutes). Since the rate of metabolism of certain neurotransmitters (e.g., glutamate) is fast, short incubation times are necessary to avoid the degradation of these substances during the uptake studies (*Balcar* and *Johnston,* 1973; *Schon* and *Kelly,* 1974a; *Hösli* and *Hösli,* 1976a). Furthermore, it has been found that the various cell types are better preserved in cultures than in slices (for refs. see *Hökfelt* and *Ljungdahl,* 1972a; *Hösli* et al., 1972a; *Ljungdahl* and *Hökfelt,* 1973b) and, therefore, culture systems are very valuable for the study of cellular and fine-structural localization of the uptake of labeled transmitter substances.

3.1. Uptake of Amino Acid Transmitters

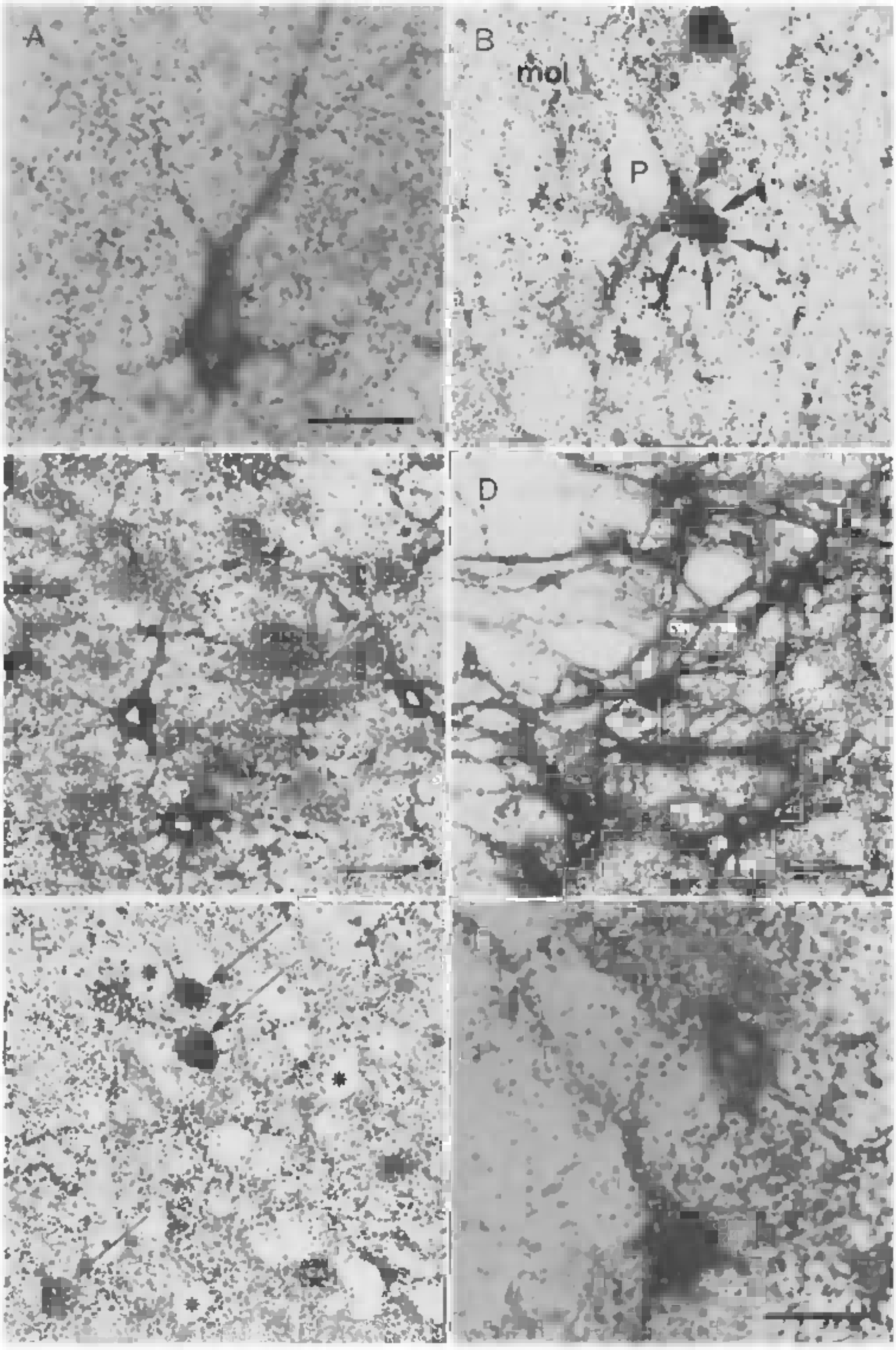
3.1.1. ^{3}H-GABA

From electrophysiologic and biochemical studies, it is suggested that Purkinje cells and cerebellar interneurones such as stellate, basket, and Golgi cells may use GABA as their transmitter (*Roberts* and *Kuriyma,* 1968; *Fonnum,* 1973; *Curtis* and *Johnston,* 1974). It is also evident that cerebellar neurones possess a specific high-affinity uptake system for this amino acid (*Iversen,* 1972; *DeFeudis,* 1975). A high-affinity uptake mechanism for GABA was also described in dispersed cell cultures of rat cerebellum (*Lasher,* 1975). Autoradiographic investigations on the cellular localization of the uptake of GABA in slices of cerebellum (*Hökfelt* and *Ljungdahl,* 1972b, c; *Iversen* and *Kelly,* 1975), in cerebellum in vivo (*Hökfelt* and *Ljungdahl,* 1972a; *Schon* and *Iversen,* 1972), and in cerebellar cultures (*Sotelo* et al., 1972; *Lasher,* 1974; *Hösli* and *Hösli,* 1976b) have revealed that a great number of neurones and glial cells accumulate the amino acid. Most of the labeled neurones appeared to be stellate cells or other cerebellar interneurones such as basket or Golgi cells (Fig. 2C). These observations were also confirmed by electron-microscopic studies, where silver grains could be visualized in neuronal cell bodies and in nerve endings of cerebellar interneurones (*Bloom* and *Iversen,* 1971; *Hökfelt* and *Ljungdahl,* 1972a; *Sotelo* et al., 1972; *Burry* and *Lasher,* 1975). Investigations using isolated large pieces of cerebellar glomeruli have demonstrated that labeled GABA as well as the GABA analogue, L-2,4-diaminobutyric acid (DABA), a substance which appears to be specifically taken up in nerve terminals but not into glial cells, were almost exclusively accumulated by Golgi axon terminals (*Wilkin* et al., 1974; *Kelly* et al., 1975).

In contrast to the findings in the cerebellum in vivo or in cerebellar slices (*Hökfelt* and *Ljungdahl,* 1972a, b, c,; *Schon* and *Iversen,* 1974; *Iversen* and *Kelly,* 1975), where Purkinje cells did not take up ^{3}H-GABA (Fig. 2B), it was observed that in cerebellar cultures (*Sotelo* et al., 1972; *Lasher,* 1974; *Hösli* and *Hösli,* 1976b) and in cerebellar transplants (*Ljungdahl* et al., 1973) there was a strong accumulation of GABA into Purkinje cells (Fig. 2A). There is convincing evidence that Purkinje cell axons which terminate on Deiter's neurones use GABA as transmitter substance (*Obata* et al., 1967; *Obata* and *Takeda,* 1969; *Bruggencate* and *Engberg,* 1971), and it is therefore surprising that Purkinje cells studied in slices or in vivo did not take up the amino acid (Fig. 2B). Since GABA is rapidly taken up by Bergman glia which surrounds the Purkinje cells (Fig. 2B) (*Hökfelt* and *Ljungdahl,* 1972a, b, c), it has been suggested that these glial cells, which might prevent the uptake of GABA into Purkinje cells in vivo, might be disturbed or absent in tissue cultures or in transplants (*Hökfelt* and *Ljungdahl,* 1972a, b, c; *Ljungdahl* and *Hökfelt,* 1973b; *Schon* and *Iversen,* 1974).

There is also much evidence from biochemical and electrophysiologic studies that GABA might act as inhibitory transmitter in the *spinal cord* (for refs. see *Curtis* and *Johnston,* 1974; *Krnjević,* 1974). High levels of GABA

Fig. 2. (A) Light-microscopic autoradiograph of a cerebellar culture after incubation with ^{3}H-GABA, $10^{-6}M$ for 2 min. The soma and dendrites of the Purkinje cell show a heavy accumulation of the amino acid. Culture: 17 days in vitro. *Bar:* 20 µm (*Hösli* and *Hösli,* 1976b). (B) Light-microscopic autoradiograph showing the grain distribution after incubation of a cerebellar slice with ^{3}H-GABA (AOAA pretreatment). Note the close correlation between grain distribution basal to the Purkinje cell body (*P*) (*arrows*), probably representing the axon terminals of basket cells. Magnification: x 500 (*Hökfelt* and *Ljungdahl,* 1972b). (C) Cerebellar culture (17 days in vitro) after incubation with ^{3}H-GABA, $10^{-6}M$ for 5 min. The cell bodies and processes of the neurones, probably stellate cells, are intensely labeled, whereas the nuclei appear to be free of label. *Bar:* 30 µm (*Hösli* and *Hösli,* 1976b). (D) Neurones of a rat brain stem culture (16 days in vitro) exhibiting a strong autoradiographic reaction after incubation with ^{3}H-GABA, $10^{-6}M$ for 5 min. *Bar:* 30 µm. (E) Light-microscopic autoradiograph of a rat spinal cord culture (26 days in vitro) incubated with ^{3}H-GABA, $10^{-6}M$ for 5 min. Several cell bodies are heavily labeled (*arrows*), whereas the majoritiy of cells (*asterisks*) are covered only by a few grains. Magnification: x 500 (*Hösli* et al., 1972a). (F) Cultured rat spinal neurone (21 days in vitro) showing a strong accumulation of ^{3}H-GABA, $10^{-6}M$, 5 min. *Bar:* 20 µm (*Hösli* et al., 1975b) ▶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were measured in the grey matter of the spinal cord, with the highest concentrations in the dorsal horns (*Graham* et al., 1967). Microelectrophoretically applied, GABA has been found to cause an inhibition of spinal interneurones (*Bruggencate* and *Engberg*, 1968) and motoneurones (*Curtis* et al., 1968). Furthermore, in chick spinal cord cultures (*Tunnicliff* et al., 1973) and in slices of cat spinal cord (*Balcar* and *Johnston*, 1973), GABA was accumulated by a high-affinity transport system. Autoradiographic investigations on the uptake of ^{3}H-GABA in the spinal cord in vivo have revealed that the amino acid was localized in small neurones and nerve terminals in the border area between laminae VI and VII of Rexed (*Rexed*, 1954) and in laminae I-III, whereas in the ventral horn comparatively few nerve endings were labeled (*Ljungdahl* and *Hökfelt*, 1973b). In electron-microscopic investigations on slices and homogenates of spinal cord, it was observed that ^{3}H-GABA was localized to a great extent in nerve terminals (*Iversen* and *Bloom*, 1972; *Ljungdahl* and *Hökfelt*, 1973b) and in glial cells (*Ljungdahl* and *Hökfelt*, 1973b). In cultures of human and rat spinal cord, ^{3}H-GABA was taken up by many neurones (Fig. 2E, F) as well as by glial cells (*Hösli* et al., 1972a, 1975b). The activity of the labeled amino acid varied considerably between individual cells. Some neurones were heavily labeled, whereas other neurones showed little or no autoradiographic reaction (Fig. 2E). It was, however, not possible to determine whether GABA is taken up by a specific neuronal population or by a specific cell type which uses this amino acid as transmitter substance (*Hösli* et al., 1972a).

Uptake of GABA by neurones and by a great number of glial cells was also observed in *brain stem* cultures (Fig. 2D) (*Hösli* and *Hösli*, unpublished observations). Electron-microscopic studies by *Iversen* and *Bloom* (1972) have also demonstrated that in homogenates of rat medulla and pons there was a relatively large proportion (25%) of labeled terminals after incubation with ^{3}H-GABA.

3.1.2. ^{3}H-Glycine

It has been proposed that glycine acts as an inhibitory transmitter substance on spinal motoneurones and interneurones (*Bruggencate* and *Engberg*, 1968; *Curtis* et al., 1968; *Werman* et al., 1968; *Aprison* et al., 1969). This amino acid was found to be present in high concentrations in the spinal cord with the highest values in the ventral grey matter (*Graham* et al., 1967; *Johnston*, 1968). Glycine caused a hyperpolarization of the cell membrane of spinal motoneurones (*Curtis* et al., 1968; *Werman* et al., 1968) and interneurones (*Bruggencate* and *Engberg*, 1968) which is associated with changes in membrane conductance similar to that of postsynaptic inhibition (*Bruggencate* and *Engberg*, 1968; *Curtis* et al., 1968; *Werman* et al., 1968; *Curtis* and

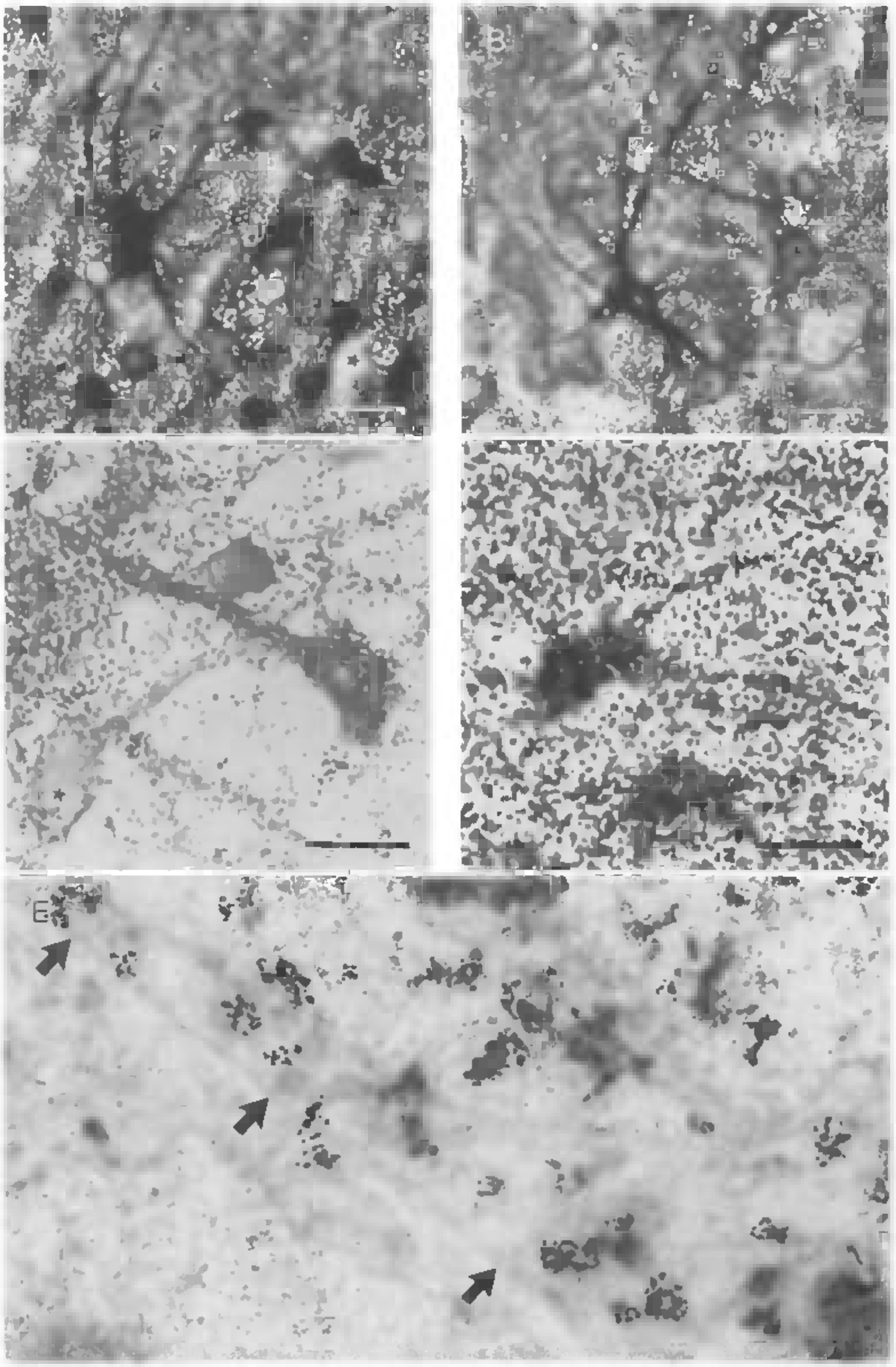
Johnston, 1974). Furthermore, a high-affinity uptake system for glycine has been found in slices and homogenates of spinal cord (*Johnston* and *Iversen,* 1971; *Neal,* 1971; *Arregui* et al., 1972; *Logan* and *Snyder,* 1972; *Balcar* and *Johnston,* 1973; *Honegger* et al., 1974; *Price* et al., 1976) as well as in cultures of chick spinal cord (*Cho* et al., 1973). Autoradiographic studies in slices and in the spinal cord in vivo have shown that ^{3}H-glycine was taken up by small to medium-sized nerve cells mainly lying in the ventral horn, the large motoneurones being almost free of label (*Hökfelt* and *Ljungdahl,* 1971b, 1972b, c; *Ljungdahl* and *Hökfelt,* 1973a, b). A particularly high density of ^{3}H-glycine was, however, described around the perikarya of motoneurones (*Matus* and *Dennison,* 1971; *Hökfelt* and *Ljungdahl,* 1971b, 1972b, c). Studies using electron-microscopic autoradiography have revealed that labeled glycine was mainly localized in nerve endings, most of which contained flat vesicles (*Matus* and *Dennison,* 1971; *Price* et al., 1976), over neuronal cell bodies, probably representing spinal interneurones, and in glial cells (*Hökfelt* and *Ljungdahl,* 1971b; *Matus* and *Dennison,* 1971, 1972; *Ljungdahl* and *Hökfelt,* 1973a, b). After incubation of human and rat *spinal cord* cultures with ^{3}H-glycine, it was observed that a great number of neurones and glial cells showed an intense autoradiographic reaction (*Hösli* et al., 1972a, 1974, 1975b). As was observed with ^{3}H-GABA, the activity of ^{3}H-glycine varied considerably between individual neurones. Some cells were intensely labeled, whereas other neurones exhibited only little or no autoradiographic reaction (Fig. 3C, E). It was not possible to determine whether glycine was taken up by the same or a different neuronal population than GABA. However, *Iversen* and *Bloom* (1972) have observed that after labeling spinal cord homogenates with a mixture of ^{3}H-GABA and ^{3}H-glycine, approximately 50% of the synaptosomes showed an autoradiographic reaction which would be the sum of the values obtained after incubation with either glycine (26%) or GABA (25%) alone. From these results, it was suggested that the two amino acids are accumulated by different synaptosomal populations (*Iversen* and *Bloom,* 1972).

Biochemical and electrophysiologic studies also provide evidence that glycine may be an inhibitory transmitter in the medulla oblongata (*Aprison* et al., 1969; *Hösli* and *Tebēcis,* 1970; *Hösli* and *Haas,* 1972). A specific high-affinity uptake mechanism for glycine has been demonstrated in slices of rat brain stem by *Johnston* and *Iversen* (1971). In autoradiographic studies on human and rat brain stem cultures, ^{3}H-glycine was found to be accumulated by a relatively large number of neurones (Fig. 3D) and by glial cells (Fig. 6F) (*Hösli* and *Hösli,* 1972; *Hösli* et al., 1975b), the uptake pattern being similar to that of ^{3}H-GABA. The uptake of both amino acids was temperature and sodium dependent (*Hösli* and *Hösli,* 1972; Hösli et al., 1975b). In organotypic cultures of rat cerebellum, only few neurones have taken up ^{3}H-glycine (*Hösli* and *Hösli,* 1976b), whereas in dissociated cerebellar cultures, most neurones showed some affinity for this amino acid (*Lasher,* 1974). However, the intensity of labeling with ^{3}H-glycine was much weaker than after incubation with ^{3}H-GABA (*Lasher,* 1974; *Hösli* and *Hösli,* 1976b). Similar results were also obtained in slices of rat cerebellum (*Hökfelt* and *Ljungdahl,* 1972c) and after intraventricular injection of ^{3}H-glycine into the cerebellum in vivo (*Schon* and *Iversen,* 1974). The findings that in spinal cord and brain stem cultures a great number of neurones were intensely labeled after incubation with ^{3}H-glycine, whereas in cerebellar cultures only a few cells showed a weak autoradiographic reaction, are consistent with biochemical studies by *Johnston* and *Iversen* (1971), demonstrating a high affinity uptake system for glycine in the spinal cord and medulla oblongata but not in other regions of the CNS.

3.1.3. L-^{3}H-Glutamic Acid and L-^{3}H-Aspartic Acid

Electrophysiologic and biochemical studies provide much evidence that glutamate and aspartate might act as excitatory transmitters in various regions of the mammalian CNS (for refs. see *Curtis* and *Johnston,* 1974; *Krnjević,* 1974). Biochemical investigations on the regional distribution of glutamate and aspartate suggest that these amino acids may function as transmitters in the mammalian spinal cord, glutamate being released by primary afferent fibers (*Graham* et al., 1967; *Duggan* and *Johnston,* 1970; *Johnson* and *Aprison,* 1970; *Johnson,* 1972) and aspartate being associated with interneurones (*Graham* et al., 1967). Both amino acids caused a depolarization of spinal neurones in vivo (*Bernardi* et al., 1972; *Curtis* et al., 1972; *Curtis* and *Johnston,* 1974) and in tissue culture (*Hösli* et al., 1973a, 1976a) which is associated with an increase in membrane conductance (*Bernardi* et al., 1972; *Curtis* et al., 1972; *Hösli* et al., 1973a, 1976a). Studies on uptake kinetics have shown that in the spinal cord and in the cerebral cortex, glutamate and aspartate are taken up by high-affinity transport systems (*Johnson* and *Aprison,* 1970; *Wofsey* et al., 1971; *Logan* and *Snyder,* 1972; *Balcar* and *Johnston,* 1973). A high-affinity uptake mechanism for glutamate was also described in cul-

Fig. 3. (A, B) Rat spinal cord cultures (18 days in vitro) after incubation with L-^{3}H-glutamic acid (A) and L-^{3}H-aspartic acid (B) ($10^{-6} M$ for 5 min). Some neurones show a heavy accumulation of silver grains over the cell bodies and processes; other neurones (*asterisks*) are almost free of label. *Bar:* 30 µm (*Hösli* and *Hösli,* 1976a). (C) Human spinal neurones (fetus 9 weeks in utero, 14 days in vitro) after incubation with ^{3}H-glycine (10^{-6} *M* for 30 s). Unlabeled cells are marked with *asterisks. Bar:* 20 µm (*Hösli* and *Hösli,* 1976a). (D) Light-microscopic autoradiograph of a rat brain stem culture (15 days in vitro) incubated with ^{3}H-glycine, $10^{-6} M$ for 5 min. Note strong accumulation of grains over the cell body and two processes. *Bar:* 20 µm (*Hösli* and *Hösli,* 1972). (E) Electron-microscopic autoradiograph of rat spinal cord culture (26 days in vitro) incubated with ^{3}H-glycine ($5 \times 10^{-6} M$, 15 min). Parts of two neurones are seen, *arrow* indicating cell border. The upper cell is covered by many grains, whereas the lower one is almost completely free of label. Magnification: x 14.300 (*Hösli* et al., 1972a) ▶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tures of chick spinal cord (*Cho* et al., 1973). Autoradiographic studies on the cellular localization of the uptake of L-^{3}H-glutamic acid and L-^{3}H-aspartic acid in cultures of human and rat spinal cord and brain stem (*Hösli* et al., 1973c, 1974, 1975b; *Hösli* and *Hösli*, 1976a) have revealed that both amino acids were accumulated by a relatively great number of large and small neurones (Figs. 3A, B, 6A) and by almost all glial cells (Figs. 6B, C, D). As was observed after incubation with inhibitory amino acids, there was also a number of neurones which were unlabeled after incubation with L-^{3}H-glutamic acid and L-^{3}H-aspartic acid, suggesting that the excitatory amino acids are taken up only by a certain population of cells (*Hösli* and *Hösli*, 1976a). This is in contrast to autoradiographic studies on the uptake of glutamate in CNS slices (*Hökfelt* and *Ljungdahl*, 1972b, c) in the retina (*Ehinger* and *Falck*, 1971; *Ehinger*, 1972), in sensory ganglia (*Schon* and *Kelly*, 1974a), and in insect neuromuscular junctions (*Faeder* and *Salpeter*, 1970), where the amino acid was only accumulated by glial elements but not by neurones. However, biochemical studies on homogenates of rat spinal cord and cortex have demonstrated that glutamate and aspartate were taken up by a unique synaptosomal fraction (*Wofsey* et al., 1971; *Logan* and *Snyder*, 1972; *Honegger* et al., 1974). Furthermore, there is evidence from autoradiographic investigations that synaptosomes are probably the most important site of uptake of L-^{3}H-glutamic acid in brain homogenates (*Beart*, 1976; *Iversen* and *Storm-Mathisen*, 1976). In contrast, *McLennan* (1976) has shown that in the cortex and thalamus of rats in vivo, L-^{3}H-glutamate was only rarely accumulated by synaptic terminals while the cell bodies of neurones and glial cells were frequently labeled.

Studies on hamster cerebellum, where a selective destruction of granule cells was obtained by a parovirus, have demonstrated that the high-affinity uptake for glutamate in synaptosomes was decreased by 65%-70% (*Young* et al., 1974). From these results, it was concluded that glutamate might be the natural neurotransmitter of cerebellar granule cells. However, uptake of glutamate in slices and in cultures of dissociated cerebellum was almost exclusively observed in glial elements (*Hökfelt* and *Ljungdahl*, 1972b, c; *Lasher*, 1974). After incubation of organotypic cultures of cerebellum with L-^{3}H-glutamic acid, it was seen that after a short incubation time all glial cells were intensely labeled, and it was, therefore, not possible to determine whether small neurones, e.g., granule cells, had also accumulated the amino acid (*Hösli* and *Hösli*, unpublished observations).

3.2. Uptake of Monoamines

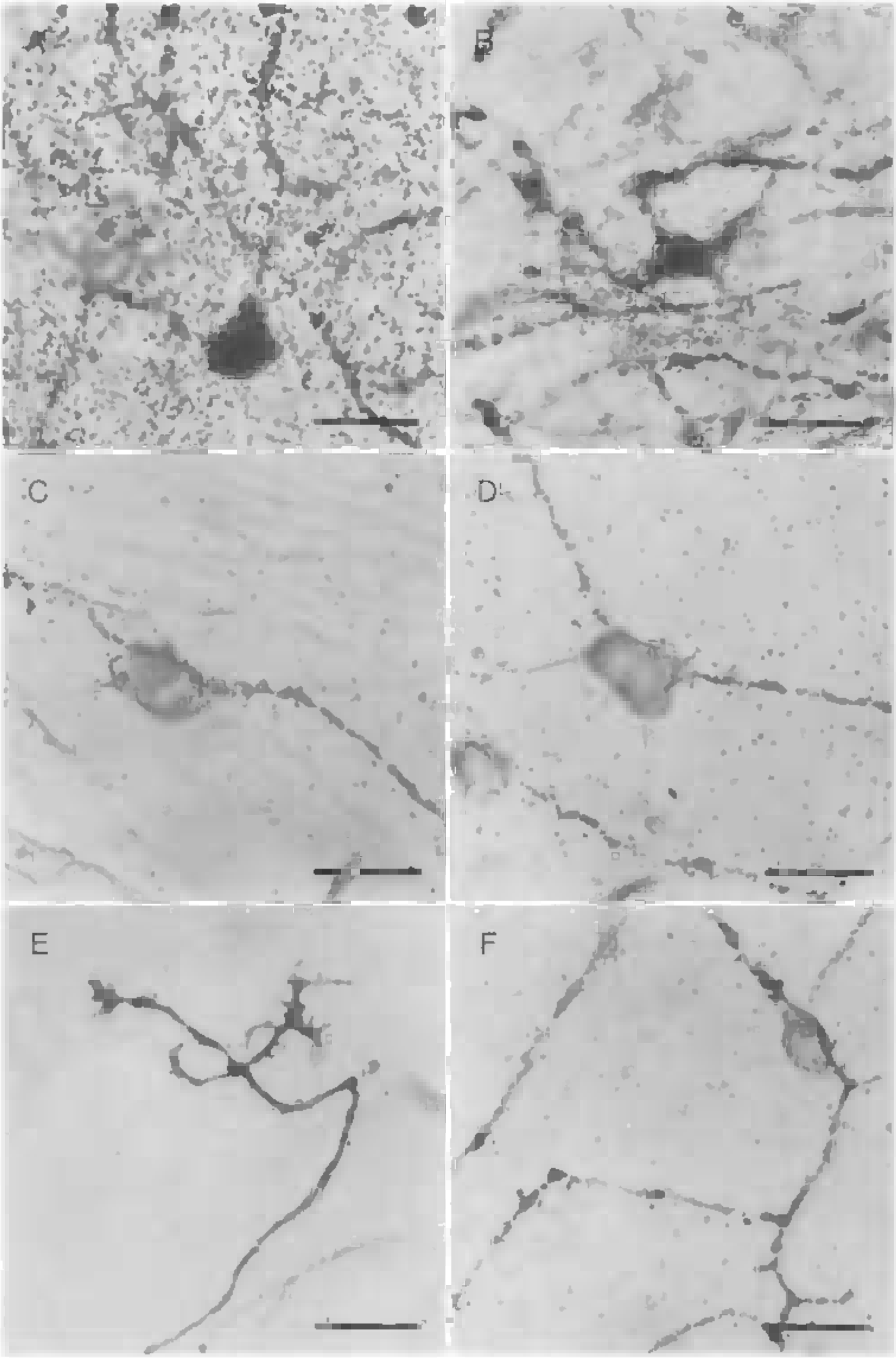
From biochemical and electrophysiologic studies, it is suggested that monoamines such as noradrenaline (NA), 5-hydroxytryptamine (5-HT), and dopamine (DA) might act as transmitter substances in the mammalian CNS (*Bertler* and *Rosengren,* 1959; *Carlsson,* 1959; *Hösli* et al., 1971c; *Krnjević*, 1974). Fluorescence microscopic investigations have shown that a great number of monoamine-containing neurones are localized in the brain stem forming part of the descending and ascending monoamine-containing pathways (*Dahlström* and *Fuxe,* 1964; *Fuxe,* 1965a, b; *Andén* et al., 1966a, b). High-affinity uptake systems for NA, 5-HT, and DA have been described in various parts of the mammalian CNS (*Snyder* et al., 1970; *Garey,* 1976). Furthermore, microelectrophoretically applied monoamines exert excitant and depressant actions in many regions of the nervous system (for ref. see *Krnjević,* 1974).

Studies on the cellular localization of the uptake of monoamines in brain slices or in the CNS in situ by using fluorescence microscopic or autoradiographic techniques have revealed that NA, DA, and 5-HT are predominantly taken up in nerve endings and unmyelinated axons (*Aghajanian* and *Bloom,* 1967; *Lenn,* 1967; *Hökfelt* and *Ljungdahl,* 1971a, 1972b, c; *Cuello* and *Iversen,* 1973; *Descarries* and *Lapierre,* 1973; *Dow* et al., 1973; *Kuhar* and *Aghajanian,* 1973; *Calas* et al., 1974; *Dow* and *Laszlo,* 1976). Autoradiographic studies in cultures of rat brain stem have demonstrated that labeled monoamines were taken up mainly by nerve fibers, by a relatively small number of neurones but not by glial cells (Fig. 4A, B) (*Hösli* et al., 1975a). Accumulation of labeled NA and 5-HT in neuronal cell bodies has also been reported in the brain stem in situ (*Fuxe* et al., 1968). Furthermore, fluorescence microscopic studies from our laboratory have revealed that neurones exhibiting a specific fluorescence for monoamines are present in the explant of cultured rat brain stem (Fig. 1F) (*Hösli* et al., 1971a, 1973e). A strong accumulation of labeled monoamines in brain stem cultures was mainly observed in nerve fibers growing out from the explant into the outgrowth zones. The monoamines were often concentrated in dot-like structures (Fig. 4F) (*Hösli* et al., 1975a), being similar to the varicosities described in fluorescence microscopic studies (*Fuxe,* 1965a, b; *Hökfelt* and *Fuxe,* 1969; *Calas* et al., 1974; *Mugnaini* and *Dahl,* 1975). Growth cones exhibiting a strong autoradiographic reaction were frequently observed at the growing tips of labeled nerve fibers (Fig. 4E). Highly fluorescent growth cones have also been described in regeneration studies of adrenergic nerves in the peripheral nervous system (*Blümcke* and *Niedorf,* 1965; *Olson* and *Malmfors,* 1970). In brain stem cultures, it was often seen that labeled fibers approached the cell body and processes of unlabeled neurones (Fig. 4C, D, F) and seemed to make contact with these cells (*Hösli* et al., 1975a). Although it was not possible from light-microscopic studies to determine whether these fibers form synaptic contacts, electron-microscopic investigations have demonstrated that axosomatic synapses and synapses *en passant* are frequently found in brain stem cultures (*Hösli* et al., 1975b).

Autoradiographic and fluorescence microscopic studies on the uptake of monoamines in cultures and transplants of sympathetic ganglia have shown that NA and DA were specifically taken up by nerve cell bodies and processes (*Burdman,* 1968; *Olson* and *Malmfors,* 1970; *Hervonen,* 1974). Furthermore, uptake of labeled NA was also described in axonal sprouts of superior cervical ganglia in organ culture (*Silberstein* et al., 1972).

It has been proposed that NA might act as a transmitter substance in the cerebellum (*Bloom* et al., 1971). Fluorescence microscopic studies have shown that NA-containing neurones lying in the locus coeruleus of the brain stem send their axons to cerebellar Purkinje cells (*Andén* et al., 1966a, b, 1967; *Hökfelt* and *Fuxe,* 1969; *Bloom* et al., 1971; *Olson* and *Fuxe,* 1971; *Mugnaini* and *Dahl,* 1975). NA reduced the firing rate of Purkinje cells in situ (*Hoffer* et al., 1971) and in tissue culture (*Gähwiler,* 1975b). The reduction of the firing rate of Purkinje cells by NA in the cerebellum in situ was accompanied by a hyperpolarization and a change in membrane conductance (*Siggins* et al., 1971). Autoradiographic investigations on the uptake of ^{3}H-NA in cerebellar cultures have demonstrated that only some nerve fibers growing out from the explant into the outgrowth zone had accumulated the monoamine (Fig. 5A, B) (*Hösli* and *Hösli,* 1976b). No uptake of ^{3}H-NA was observed in neuronal cell bodies or in glial cells (Fig. 5B). These findings are consistent with fluorescence histochemical studies in the cerebellum in situ, where only fluorescent nerve fibers but no cell bodies have been detected (*Andén* et al., 1966a, b, 1967; *Hökfelt* and *Fuxe,* 1969; *Bloom* et al., 1971; *Mugnaini* and *Dahl,* 1975). As was observed in brain stem cultures, the uptake of NA in the nerve fibers in cerebellar cultures was frequently concentrated in dot-like structures (Fig. 5A, D, *Hösli* and *Hösli,* 1976b) resembling the varicosities described in fluorescence histochemical studies (*Hökfelt* and *Fuxe,* 1969; *Mugnaini* and *Dahl,* 1975). Autoradiographic investigations in the cerebellum in vivo and in slices have demonstrated that ^{3}H-NA was mainly localized in fine unmyelinated axons making synap-

Fig. 4 A - F. Light-microscopic autoradiographs of rat brain stem cultures after incubation ▶ with ^{3}H-NA, ^{3}H-DA or ^{3}H-5-HT. *Bars:* 20 μm for A, B, C, D, F; 40 μm for E. (A) Neurone showing a strong autoradiographic reaction of the cell body and processes after incubation with ^{3}H-NA for 2 min (culture 22 days in vitro) (*Hösli* et al., 1975a). (B) Intensely labeled neurone of a 16-day-old brain stem culture (incubation with ^{3}H-5-HT, 10^{-6} *M,* for 5 min) (*Hösli* et al., 1975a). (C) An intensely labeled nerve fiber appears to form contact with the cell body of an unlabeled neurone. Brain stem culture, 16 days in vitro, after incubation with ^{3}H-5-HT (*Hösli* et al., 1975a). (D) Brain stem neurone (culture 22 days in vitro) which contains no silver grains. The heavily labeled processes are probably axons from other neurones which seem to make contacts with the unlabeled cell. Incubation with ^{3}H-DA, 10^{-6} *M,* 5 min. (E) Growth cones showing an intense autoradiographic reaction after incubation with ^{3}H-5-HT, 10^{-6} *M* for 5 min. Brain stem culture, 16 days in vitro. (F) Labeled nerve fibers in the outgrowth zone of a brain stem culture (22 days in vitro) after incubation with ^{3}H-5-HT, 10^{-6} *M,* 5 min. The monoamine is concentrated in small dots giving the appearance of varicosities (*Hösli* et al., 1975a)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tic contacts with the soma and/or dendrites of Purkinje cells (*Bloom* et al., 1971). In cerebellar cultures, it was also found that labeled nerve fibers appeared to make contacts with the soma and/or processes of unlabeled neurones (Fig. 5C, D), most of which seemed to be Purkinje cells (*Hösli* and *Hösli,* 1976b). The origin of the labeled fibers in cerebellar cultures is not clear, since most of the NA-containing fibers which arise from the locus

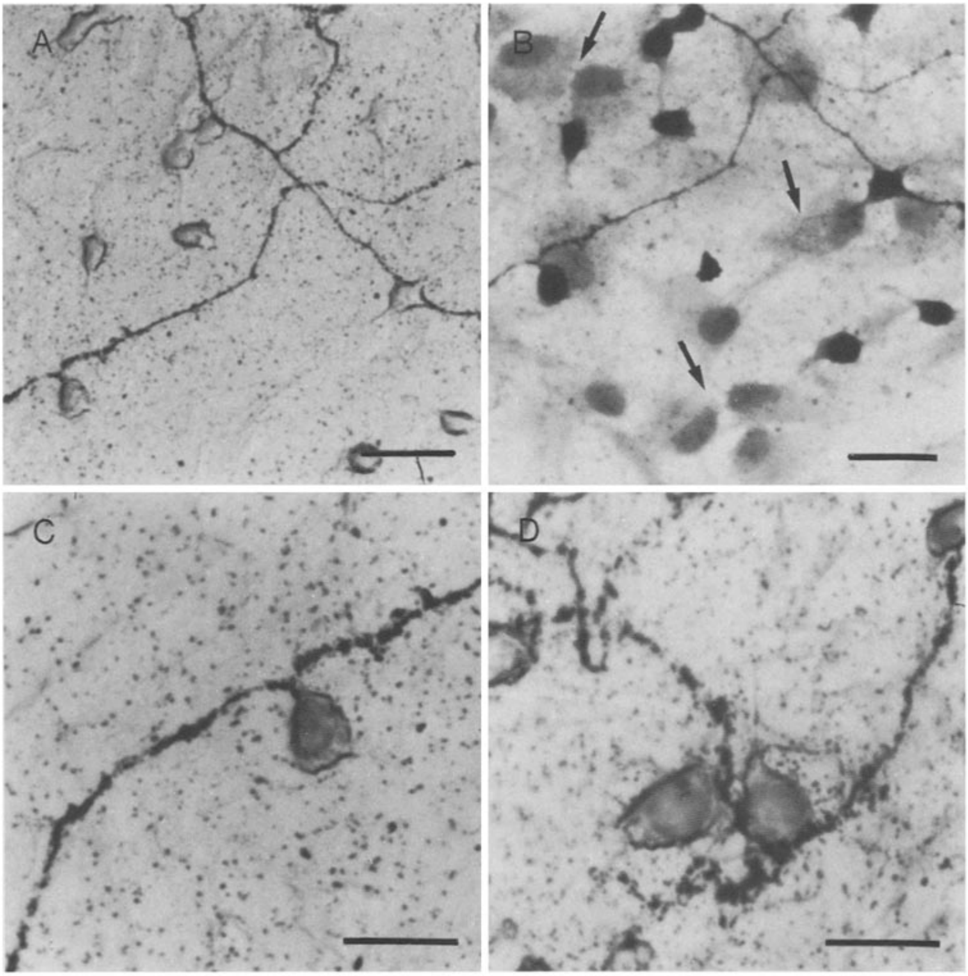


Fig. 5. (A) Autoradiograph of a cerebellar culture (19 days in vitro) after incubation with ^{3}H-NA, $10^{-6} M$ for 5 min. Intensely labeled nerve fibers are passing unlabeled neurones, most of which appear to be Purkinje cells. *Bar:* 30 μm (*Hösli* and *Hösli,* 1976b). (B) Same autoradiograph as shown in A after staining with toluidine blue for 2 min. The glial cells (*arrows*) which are now visible did not accumulate ^{3}H-NA. *Bar:* 30 μm (*Hösli* and *Hösli,* 1976b). (C) Higher magnification of an unlabeled Purkinje cell illustrated in A. The labeled fiber seems to make contact with the dendrites of this cell. *Bar:* 20 μm (*Hösli* and *Hösli,* 1976b). (D) Unlabeled neurones, probably Purkinje cells, being surrounded by intensely labeled fibers. Cerebellar culture, 19 days in vitro after incubation with ^{3}H-NA, $10^{-6} M$ for 5 min. *Bar:* 20 μm.

coeruleus (*Andén* et al., 1966a, b, 1967; *Hökfelt* and *Fuxe,* 1969; *Olson* and *Fuxe,* 1971) are probably degenerated in cultures grown for more than 2 weeks in vitro.

From these results, it is clear that the pattern of the cellular localization of the uptake of monoamines is quite different from that of amino acids. Amino acids are taken up by a relatively large number of neurones and by almost all glial elements, whereas monoamines are only accumulated in nerve fibers and in a few neurones, but not in glial cells.

3.3. Uptake of Neurotransmitters Into Glial Cells

Biochemical and autoradiographic investigations on the uptake of amino acid transmitters have shown that GABA, glycine, glutamate, and aspartate are not only taken up by neuronal cell bodies and nerve endings but to a great extent also by glial cells (*Faeder* and *Salpeter,* 1970; *Ehinger* and *Falck,* 1971; *Hamberger,* 1971; *Henn* and *Hamberger,* 1971; *Hökfelt* and *Ljungdahl,* 1971b; *Orkand* and *Kravitz,* 1971; *Ehinger,* 1972; *Hösli* and *Hösli,* 1972, 1976a, b; *Hösli* et al., 1972a, 1973c, 1974, 1975b; *Lasher,* 1974, 1975; *Faivre-Baumann* et al., 1974; *Henn* et al., 1974; *Hutchison* et al., 1974; *Schon* and *Iversen,* 1974; *Schon* and *Kelly,* 1974a, b; *Snodgrass* and *Iversen,* 1974; *Iversen* and *Kelly,* 1975; *Schubert,* 1975; *Sellström* and *Hamberger,* 1975; *Logan,* 1976). It has been reported that glial uptake has characteristics of a high-affinity transport system similar to that into neurones (*Henn* and *Hamberger,* 1971; *Faivre-Baumann* et al., 1974; *Henn* et al., 1974; *Hutchison* et al., 1974; *Schon* and *Kelly,* 1974a, b; *Schrier* and *Thompson,* 1974; *Snodgrass* and *Iversen,* 1974; *Iversen* and *Kelly,* 1975; *Lasher,* 1975). Furthermore, biochemical studies on amino acid uptake into neuronal and glial fractions by *Hamberger* (1971) have revealed that "amino acid uptake occurred at a higher rate in the glial cells than in the neuronal cells."

Cultures of CNS tissue provide an excellent tool for performing autoradiographic studies on the uptake of amino acids into glial cells, since glial cells are much better preserved in cultures than in brain slices (*Hösli* et al., 1972a). Autoradiographic studies of the uptake of GABA, glycine, glutamate, and aspartate in human and rat CNS cultures have shown that the amino acids are accumulated by the soma and processes of almost all glial cells (Fig. 6B-F), most of which appear to be protoplasmic astrocytes (*Hösli* and *Hösli,* 1972, 1976a, b; *Hösli* et al., 1973c, 1974, 1975b). This is in contrast to the uptake pattern observed in neurones, where only certain cells showed an autoradiographic reaction, whereas other neurones were unlabeled (Fig. 2E, 3A, B, C, E) (*Hösli* and *Hösli,* 1972, 1976a, b; *Hösli* et al., 1972a, 1973c, 1974, 1975b). There was also a difference in time of the uptake of the amino acids into neurones and glial cells. After short incubation times (30 s-

5 min), neurones taking up the amino acids were much more intensely labeled than glial cells, whereas after longer incubation times (10 min) glial cells also revealed a strong autoradiographic reaction (*Hösli* and *Hösli,* 1972, 1976a; *Hösli* et al., 1973c, 1975b). Similar results have been obtained by *Schon* and *Kelly* (1974b) demonstrating that the rate of GABA uptake in satellite cells of sensory ganglia is much slower than in cortical neurones. From these findings, it is suggested that different transport systems might be involved in the uptake of the amino acids in neurones and in glial cells.

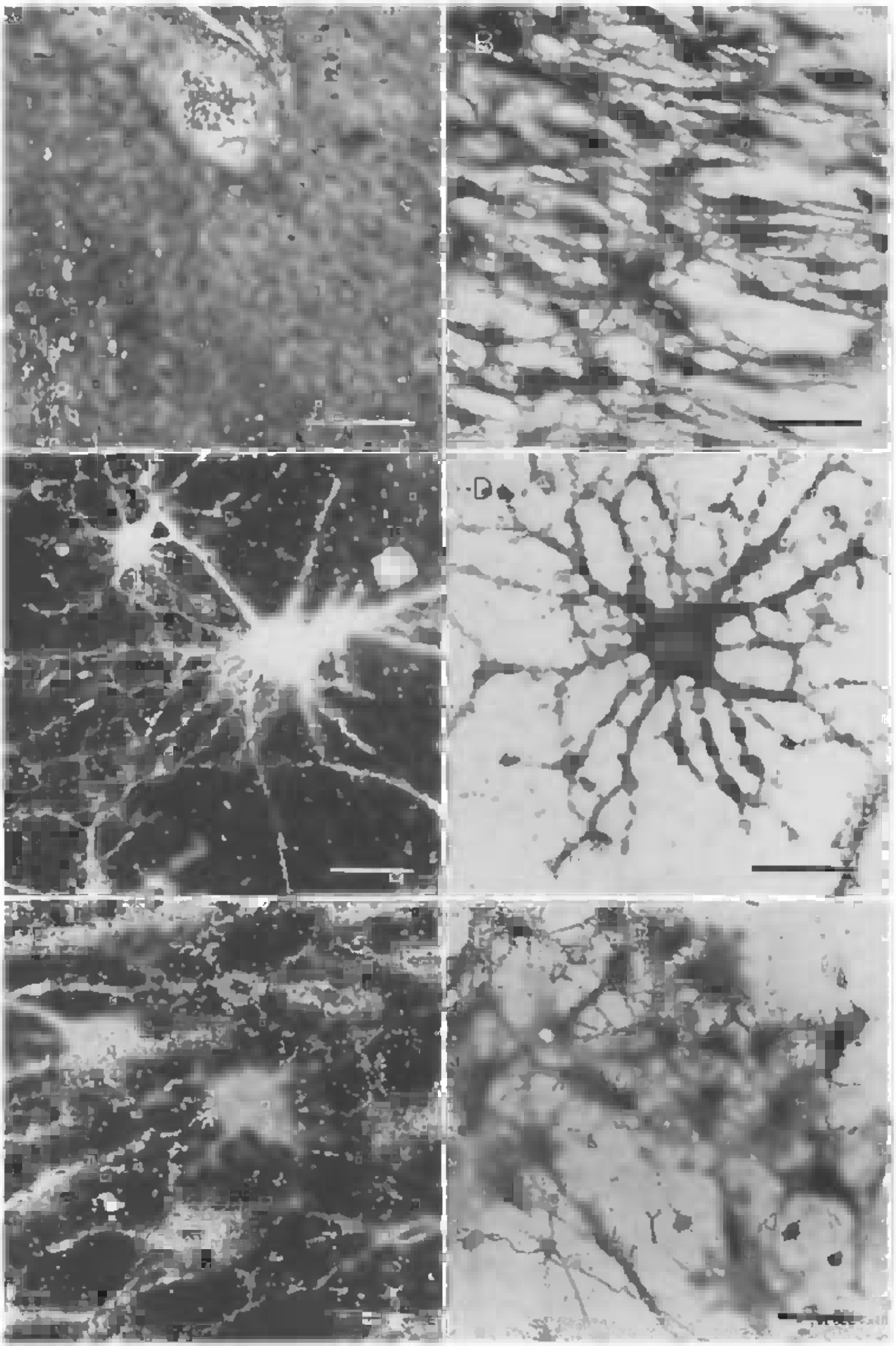
In contrast to the results obtained with amino acids, monoamines were not accumulated by glial cells in cultures of rat brain stem and cerebellum (Fig. 5B) (*Hösli* et al., 1975a; *Hösli* and *Hösli,* 1976b), although biochemical investigations on rabbit brain fractions have revealed that a small amount of 5-HT, NA, and DA was also taken up by the glial cell fraction (*Henn* and *Hamberger,* 1971). Accumulation of 5-HT into non-neuronal elements (glial cells and ependyma of the central canal) was reported by *Dow* et al. (1973) in the area postrema of the rabbit, whereas in the brain stem and in other parts of the brain no uptake of 5-HT into glial cells could be detected (*Dow* and *Laszlo,* 1976).

These results demonstrate that there is a great difference of the glial uptake pattern between monoamines and that of amino acid transmitters, where glial cells seem to play a role in transmitter inactivation.

4. Electrophysiologic Properties of Cultured Neurones

The method of tissue culture provides an excellent tool for correlating bioelectric and cytologic studies of CNS tissue in vitro, allowing microelectrode recordings from single cells under direct visual control (Fig. 7A, B) (*Hild* and

Fig. 6. (A) Human spinal cord culture (fetus 9 weeks in utero, 18 days in vitro) after incubation with L-^{3}H-glutamic acid ($10^{-6}M$ for 5 min). Labeled neurones and glial cells are found in the dense zones of the culture as well as in the outgrowth zones. *expl.* = explant. *Bar:* 200 μm (*Hösli* and *Hösli,* 1976a). (B) Labeled glial cells forming a network in the outgrowth zone of a human spinal cord culture (fetus 9 weeks in utero, 18 days in vitro) after incubation with L-^{3}H-glutamic acid ($10^{-6}M$ for 10 min). *Bar:* 50 μm (*Hösli* and *Hösli,* 1976a). (C) Dark field illumination of intensely labeled astrocytes of a rat spinal cord culture (28 days in vitro) after incubation with L-^{3}H-glutamic acid ($10^{-6}M$ for 5 min). *Bar:* 30 μm (*Hösli* and *Hösli,* 1976a). (D) Astrocyte showing a strong accumulation of L-^{3}H-aspartic acid ($10^{-6}M$ for 10 min). Rat spinal cord culture, 28 days in vitro. *Bar:* 20 μm (*Hösli* and *Hösli,* 1976a). (E) Intensely labeled glial cells in the outgrowth zone of an 18-day-old cerebellar culture after incubation with ^{3}H-GABA, $10^{-6}M$ for 10 min. Dark-field illumination. *Bar:* 30 μm (*Hösli* and *Hösli,* 1976b). (F) Autoradiograph of a brain stem culture incubated with ^{3}H-glycine, $10^{-6}M$ for 15 min. Labeled astrocytes forming a network in the outgrowth zone (culture 15 days in vitro). *Bar:* 30 μm (*Hösli* and *Hösli,* 1972) ▶

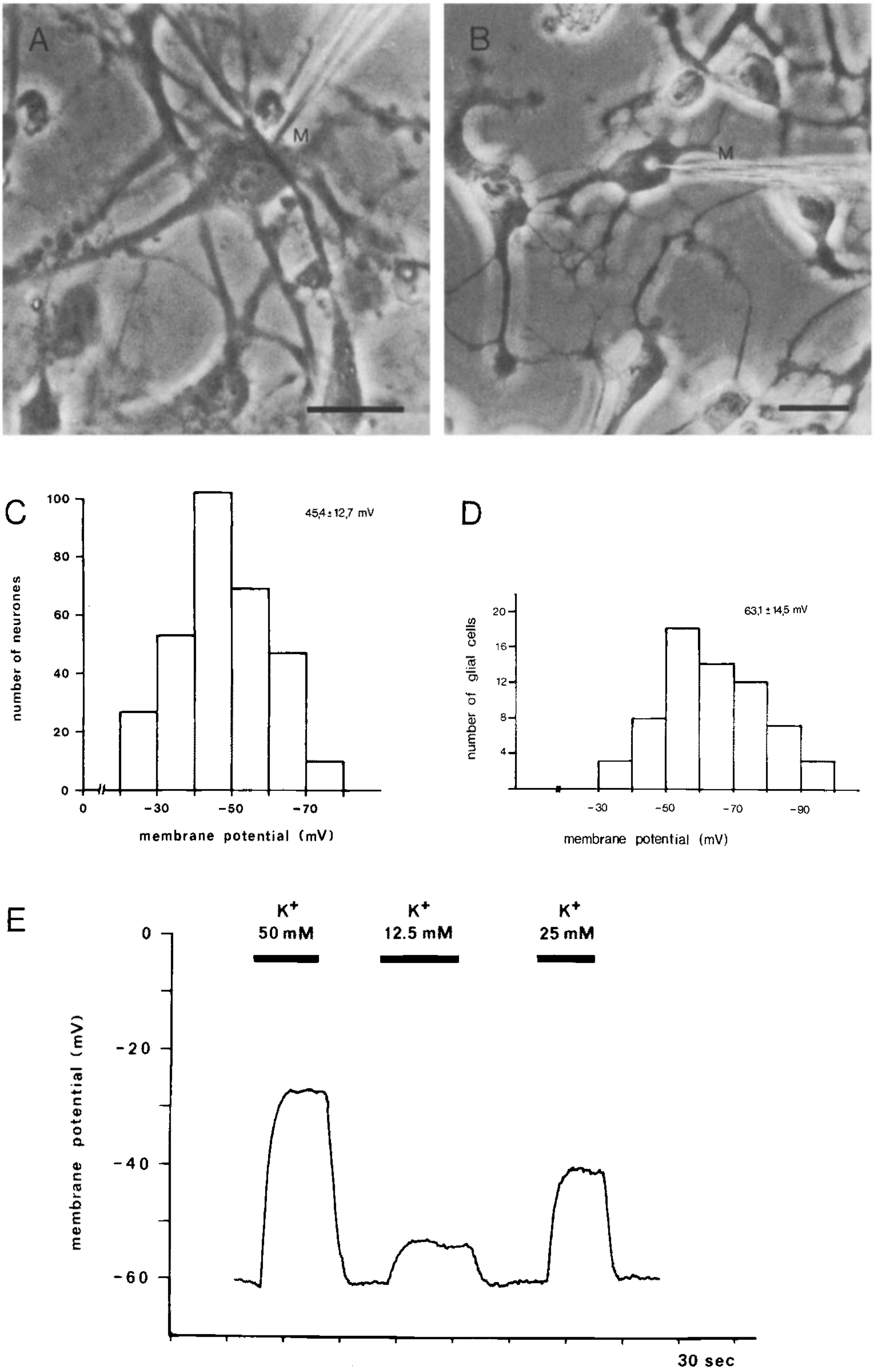

B
D

Tasaki, 1962; *Crain,* 1966, 1975; *Hösli* et al., 1971b,1975b,1976a; *Nelson,* 1975b; *Ransom* and *Nelson,* 1975). From a great number of studies on cultured neurones and glial cells, it is evident that nervous tissue in culture has to a great extent physiologic properties similar to nervous tissue in situ. Extensive reviews concerned with electrophysiologic properties of cultured nervous tissue have been written by *Crain* (1966, 1975) and by *Nelson* (1975b).

4.1. Membrane Potentials and Membrane Resistance

The first intracellular recordings from cultured neurones have been made by *Crain* (1956) from chick embryo spinal ganglion cells and later from cerebellar neurones by *Hild* and *Tasaki* (1962). The membrane potentials obtained from spinal ganglion cells ranged from -50 to -65 mV (*Crain,* 1956), and those from cerebellar neurones were -50 mV and less (*Hild* and *Tasaki,* 1962). *Peacock* et al. (1973), recording from dissociated cells of fetal mouse spinal cord, observed that resting potentials of dorsal root ganglion cells (average -51 mV) were higher than those of spinal cord neurones (average -40 mV). Microelectrode studies from our laboratory on neurones in organotypic cultures of rat and fetal human spinal cord revealed considerable differences in membrane potentials between individual neurones ranging from -25 to -74 mV with an average of -44.2 mV ± 9.8 mV for human spinal neurones and of -45.4 mV ± 12.7 mV for rat spinal neurones (*Hösli* et al., 1974). The frequency distribution of membrane potentials of rat spinal neurones is illustrated in the histogram in Figure 7C (*Hösli* et al., 1974). The great variability in membrane potentials might be due to differences in the morphologic properties of the cultured cells (vulnerability of the cell membrane, location of the cells in the culture) or to technical difficulties associated with intracellular recordings. As has been described by many authors, it was often difficult to impale cultured neurones with microelectrodes and to record stable membrane potentials over a long period of time (*Crain,* 1956; *Hild* and *Tasaki,* 1962; *Hösli* et al., 1971b, 1974, 1976a). There was often a progressive decrease of the membrane potential a few seconds after impalement, which was usually accompanied by morphologic signs of deterioration of the cell tested (*Hild* and

Fig. 7. (A) Neurone of a 28-day-old spinal cord culture (human fetus 17 weeks in utero). ▶ The tip of the glass microelectrode (*M*) is placed near the soma of the cell. Phase contrast. *Bar:* 30 μm (*Hösli* et al., 1973d). (B) Glial cell lying in the outgrowth zone of a rat spinal cord culture (28 days in vitro) after impalement by a microelectrode (*M*). Phase contrast. *Bar:* 20 μm (*Hösli* et al., 1976b). (C, D) Histograms showing the distribution of membrane potentials of neurones (C) and glial cells (D). (C: *Hösli* et al., 1974; D: *Hösli* et al., 1976b). (E) Effects of an increase of the extracellular potassium concentration on the membrane potential of a cultured spinal neurone. Duration of perfusion with high potassium concentration is represented by horizontal bars above tracings. Values of potassium are indicated in m*M* (*Hösli* et al., 1972b)


A
M
B
M
C
number of neurones
100
80
60
40
20
0
0
-30
-50
-70
membrane potential (mV)
45,4 ± 12,7 mV
D
number of glial cells
20
16
12
8
4
-30
-50
-70
-90
membrane potential (mV)
63,1 ± 14,5 mV
E
membrane potential (mV)
0
-20
-40
-60
K+ 50 mM
K+ 12.5 mM
K+ 25 mM
30 sec

Tasaki, 1962; *Hösli* et al., 1971b, 1976a). Some difficulties in penetrating cultured neurones with microelectrodes might also be explained by the fact that astroglial layers often cover the neurones (*Guillery* et al., 1970; *Hösli* et al., 1975b).

The membrane resistance of cultured spinal neurones ranged from 2 - 15 MΩ (*Hösli* et al., 1970, 1971b, 1972c; *Peacock* et al., 1973; *Hooisma* et al., 1975). As was observed on other excitable cells (for ref. see *Hösli* et al., 1976a), an increase of external potassium concentration caused a depolarization of the cell membrane of cultured neurones (Fig. 7E) which was proportional to the concentration of potassium in the bathing fluid (*Hild* et al., 1958; *Zhukovskaya* and *Chailakhyan,* 1970; *Hösli* et al., 1972b, 1974).

4.2. Action Potentials and Synaptic Potentials

Action potentials, either spontaneous or evoked by electric stimulation, have been recorded extracellularly and intracellularly from neurones of organotypic and dissociated cultures of the spinal cord (*Crain* and *Peterson,* 1964; *Crain,* 1966, 1975; *Hösli* et al., 1972c, 1974, 1976a; *Peacock* et al., 1973; *Fischbach* and *Dichter,* 1974; *Hooisma* et al., 1975; *Ransom* and *Nelson,* 1975), of the brain stem (*Crain* and *Peterson,* 1975; *Hösli* et al., unpublished observations), of the cerebellum (*Hild* and *Tasaki,* 1962; *Schlapfer,* 1969; *Gähwiler* et al., 1972; *Leiman* and *Seil,* 1973; *Nelson* and *Peacock,* 1973; *Calvet,* 1974; *Geller* and *Woodward,* 1974; *Leiman* et al., 1975; *Gähwiler,* 1976), of the hippocampus (*Shtark* et al., 1976), of the olfactory bulb (*Corrigall* et al., 1976), of the cerebral cortex (*Crain* and *Bornstein,* 1964; *Crain,* 1966, 1975; *Godfrey* et al., 1975), and of dorsal root (*Scott* et al., 1969; *Varon* and *Raiborn,* 1971; *Peacock* et al., 1973; *Obata,* 1974; *Lawson* et al., 1976; *Hösli* et al., unpublished observations) and vegetative ganglia (*Crain,* 1971; *Chalazonitis* et al., 1974; *Obata,* 1974; *Ko* et al., 1976).

The spontaneous spike activity of cultured neurones was dependent on the temperature of the bathing fluid (*Crain,* 1966; *Gähwiler* et al., 1972; *Hösli* et al., unpublished observations). There was a progressive but reversible decrease of the firing frequency by lowering the temperature of the bathing solution from 37°C to 10°C. Most neurones stopped firing between 20°C and 10°C, although some cells were still spontaneously firing at 5°C (*Crain,* 1966; *Gähwiler* et al., 1972). An increase in temperature to 42°C irreversibly stopped the spontaneous activity of all cells.

There is good evidence from electrophysiologic studies indicating the existence of functional synapses in nervous tissue in culture (for refs. see *Crain,* 1966, 1975; *Nelson* 1975a, b; *Ramson* and *Nelson,* 1975; *Varon,* 1975). Inhibitory as well as excitatory postsynaptic potentials have been recorded from neurones of dissociated mouse cerebellum (*Nelson* and

Peacock, 1973), of hippocampal explants (*Zipser* et al., 1973), and of organotypic and dissociated spinal cord cultures (*Hösli* et al., 1972c, 1974; *Fischbach* and *Dichter,* 1974; *Nelson,* 1975b, *Ransom* and *Nelson,* 1975).

5. Electrophysiologic Properties of Cultured Glial Cells

Since there are considerable difficulties in identifying glial cells in situ on the basis of morphologic and electrophysiologic properties, the technique of tissue culture provides a unique opportunity to perform intracellular recordings from glial cells under direct visual control, allowing the identification of the different cell types by morphologic criteria (*Hild* et al., 1958; *Wardell,* 1966; *Klee* and *Hild,* 1967; *Walker* and *Hild,* 1969; *Hösli* et al., 1970, 1976b, *Trachtenberg* et al., 1972; *Vernadakis* and *Berni,* 1973; *Kukes* et al., 1976).

As was experienced with intracellular recordings from cultured neurones, it was often difficult to impale glial cells in tissue culture with microelectrodes and to maintain stable membrane potentials over a period of minutes. Although the size of the glial cells was usually smaller than that of the neurones studied, it was often easier to maintain stable membrane potentials from astrocytes than from neurones (*Hild* and *Tasaki,* 1962; *Hösli* et al., 1974, 1975b, 1976b). The technical difficulties associated with microelectrode recordings as well as the use of different types of cultures might explain the extremely wide scatter of membrane potentials recorded from cultured glial cells by different laboratories. The membrane potentials of glial cells described by various authors ranged from -4 mV to -90 mV (*Hild* et al., 1958; *Hild* and *Tasaki,* 1962; *Wardell,* 1966; *Klee* and *Hild,* 1967, *Walker* and *Hild,* 1969; *Trachtenberg* et al., 1972; *Vernadakis* and *Berni,* 1973; *Hösli* et al., 1974, 1976b; *Hamprecht* et al., 1976; *Kukes* et al., 1976). *Trachtenberg* et al. (1972) obtained only very low resting potentials (average -7.7 mV) from cultured glial cells of fetal human cortex.

The membrane potentials recorded in our laboratory from glial cells in cultures of fetal human and rat spinal cord were usually higher than those obtained from cultured neurones. Figure 7D shows a histogram illustrating the distribution of membrane potentials of glial cells of cultured rat spinal cord ranging from -36 mV to -90 mV with a mean value of -63.1 mV ± 14.5 mV, whereas the potentials recorded from neurones under similar culture conditions (Fig. 7C) ranged from -25 mV to -72 mV with an average of -45.4 mV ± 12.7 mV (*Hösli* et al., 1974, 1976b). These results are consistent with observations made on glial cells of the mammalian (*Coombs* et al., 1955a; *Krnjević* and *Schwartz,* 1967; *Grossman* and *Hampton,* 1968; *Dennis* and *Gerschenfeld,* 1969) and of the leech CNS in situ (*Kuffler* and *Nicholls,* 1966), demonstrating that glial cells usually had higher resting potentials than neurones.

The input resistance of cultured glial cells also varied considerably between individual cells and measurements by different laboratories, ranging from 0.5 - 14 MΩ (*Hild* and *Tasaki,* 1958; *Wardell,* 1966; *Hösli* et al., 1970; *Trachtenberg* et al., 1972; *Kukes* et al., 1976).

As was observed on cultured neurones, an increase of the potassium concentration in the extracellular fluid caused a marked depolarization of the cell membrane of glial cells (*Hild* and *Tasaki,* 1958; *Wardell,* 1966; *Hösli* et al., 1974; *Hamprecht* et al., 1976; *Kukes* et al., 1976). Studies by *Kuffler* and *Nicholls* (1966) in the leech CNS, relating the change of the resting potentials of glial cells to the potassium concentration in the bathing fluid, suggest that glial cells behave like a potassium electrode over a wide range of extracellular potassium concentrations.

6. Action of Neurotransmitters on Cultured Neurones

6.1. Effects of Inhibitory Amino Acids

Biochemical and electrophysiologic studies indicate that the amino acids glycine, GABA, and taurine may act as inhibitory transmitter substances in the mammalian CNS (for refs. see *Werman,* 1972; *Curtis* and *Johnston,* 1974; *Krnjević,* 1974; *Hösli* et al., 1975b). Microelectrophoretically applied, glycine, GABA, and taurine caused a depression of the firing rate of neurones in many regions of the CNS. Intracellular studies revealed a hyperpolarizing action of these amino acids, associated with a marked increase in membrane conductance (*Bruggencate* and *Engberg,* 1968; *Curtis* et al., 1968; *Werman* et al., 1968; *Hösli* and *Haas,* 1972; *Hösli* et al., 1973b; *Krnjević, 1974).*

In contrast to microelectrophoretic studies performed in situ, the method of tissue culture allows an exact location of the drug-administering micropipette on different parts of the neuronal membrane under direct visual control (Fig. 8A) (*Hösli* et al., 1971b, 1973a). Furthermore, it is possible to study the effect of the amino acids in exact concentrations by adding them to the bathing fluid (*Hösli* et al., 1973a, d, 1976a; *Ransom* and *Nelson,* 1975). Glycine applied microelectrophoretically or added to the bathing solution in concentrations of 10^{-3} to 10^{-5} *M* caused a hyperpolarization of cultured spinal (Fig. 8C, 9A, 10K) (*Hösli* et al., 1971b, 1973c, 1974, 1975b; *Ransom* and *Nelson,* 1975, *Ransom* and *Barker,* 1975) and cortical neurones (*Godfrey* et al., 1975) which was accompanied by a reduction of the membrane resistance similar to that observed in neurones in situ (*Bruggencate* and *Engberg,* 1968; *Curtis* et al., 1968; *Werman* et al., 1968). Similar results have also been obtained after administration of GABA and taurine (Fig. 9A)

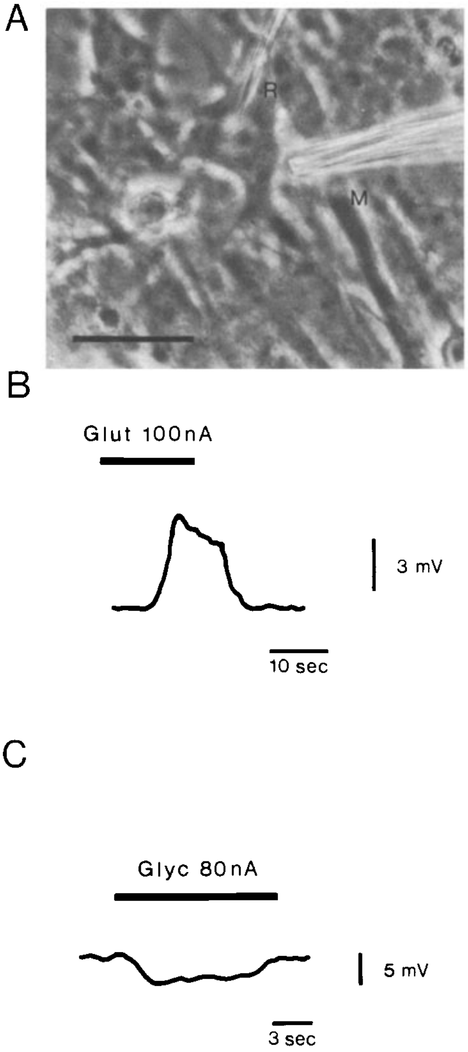


Fig. 8. (A) Phase contrast picture of a rat spinal neurone in tissue culture (23 days in vitro). *R*, recording microelectrode; M: four-barrel micropipette for the microelectrophoretic administration of the neurotransmitters. *Bar:* 30 μm (*Hösli* et al., 1973a). (B) Depolarizing effect of microelectrophoretically administered glutamate (*Glut*, ejecting current 100 nA) on a rat spinal neurone (25 days in vitro) (*Hösli* et al., 1971b). (C) Hyperpolarizing action of microelectrophoretically administered glycine (*Glyc*, ejecting current 80 nA) on another rat spinal neurone (21 days in vitro). Time: 10 s (B) and 3 s (C). Duration of drug application is indicated by horizontal bar above tracings. *Ordinate:* membrane potential in mV (*Hösli* et al., 1973a)

(*Godfrey* et al., 1975; *Hösli* et al., 1975b; *Ransom* and *Nelson*, 1975; *Bonkowsky* and *Dryden*, 1976). A comparison of the hyperpolarizing effects of glycine and taurine on spinal and brain stem neurones in tissue culture (*Hösli* et al., 1975b) with that of spinal and brain stem neurones of the cat in situ (*Hösli* and *Haas*, 1972; *Hösli* et al., 1973b; *Curtis* and *Johnston*, 1974) indicate that cultured neurones possess receptors which are influenced by these amino acids in a way similar to those studied in vivo (Fig. 9).

When glycine and GABA were tested on the electric activity of cultured neurones by extracellular recording methods, it was observed that both substances caused a marked decrease of the firing rate of cerebellar neurones (*Geller* and *Woodward*, 1974; *Gähwiler*, 1975a, 1976). Furthermore, studies on fetal spinal cord explants have revealed a "selective depression of major components of complex synaptic network discharges" in high glycine-containing (10^{-3} *M*) bathing solution (*Crain,* 1974). As has been described in vivo (*Curtis* et al., 1968; *Curtis* and *Johnston,* 1974; *Krnjević*, 1974), the convulsant strychnine selectively antagonized the depressant action of glycine on cultured spinal (*Crain,* 1974) and cerebellar neurones (*Gähwiler,* 1976), whereas the depression caused by GABA of cultured spinal, cortical, and cerebellar neurones was blocked by bicuculline and picrotoxin (*Geller* and *Woodward,* 1974; *Crain,* 1975; *Gähwiler,* 1975a; *Bonkowsky* and *Dryden,* 1976). Recent investigations on olfactory bulb explants in tissue culture have shown that the slow-wave discharges evoked in these cultures are depressed by GABA and that this depression is antagonized by bicuculline and picrotoxin (*Corrigall* et al., 1976).

There is much evidence that the hyperpolarization produced by glycine, GABA, and taurine (*Bruggencate* and *Engberg,* 1968; *Curtis* et al., 1968; *Werman* et al., 1968; *Hösli* and *Haas,* 1972; *Hösli* et al., 1973b, c; *Krnjević*, 1974) as well as that of postsynaptic inhibition (*Coombs* et al., 1955b; *Eccles,* 1957, 1964) is associated with an increase in chloride permeability. Studies on ionic mechanisms associated with the action of inhibitory transmitters

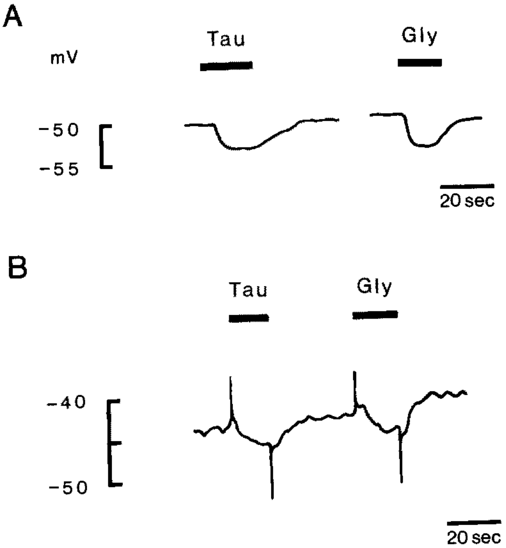


Fig. 9. (A) Hyperpolarization by taurine (*Tau*) und glycine (*Gly*) of a rat spinal neurone in tissue culture (25 days in vitro). The amino acids were added to the bathing fluid at a concentration of 10^{-3}*M*. (B) Action of taurine (*Tau*) and glycine (*Gly*) on the membrane potential of a neurone of the medulla oblongata of the cat in situ. For these studies, a combined electrode was used, consisting of a single-barrel micropipette for intracellular recording and a four-barrel micropipette from which the amino acids were administered extracellularly by microelectrophoresis. The ejecting current was 100 nA. Duration of amino acid application is indicated by horizontal bar above tracings. *Ordinate:* membrane potential in mV; *abscissa:* time, 20 s (*Hösli* et al., 1975b)

on CNS neurones in situ were made by injecting various ions into the interior of the cells, thus altering the ionic concentration gradients (*Curtis* et al., 1968; *Werman* et al., 1968; *Krnjević*, 1974). Injection of chloride ions into spinal motoneurones of the cat reversed the hyperpolarizing action of glycine (Fig.10A-D) and GABA as well as the postsynaptic potential (Fig.10E-H) to a depolarization (*Curtis* et al., 1968; *Werman* et al., 1968). Tissue culture techniques which allow the alteration of the ionic composition of the extracellular environment have proved to be an excellent tool for investigating ionic mechanisms associated with transmitter actions on neurones of the mammalian CNS. When the effect of glycine was tested on the membrane potential of cultured spinal neurones after removal of chloride ions from the extracellular fluid, the amino acid had no more a hyperpolarizing action as in normal bathing solution (Fig. 10K) but caused a marked depolarization of the cell membrane (Fig.10L, *Hösli* et al., 1973c), indicating that glycine alters the chloride permeability of spinal neurones in tissue culture as it does on spinal motoneurones in situ (*Curtis* et al., 1968; *Werman* et al., 1968).

The equilibrium potentials for the glycine and GABA hyperpolarization of cultured spinal neurones varied considerably, ranging from -30 mV to -80 mV (*Ransom* and *Nelson,* 1975).

There is considerable evidence that presynaptic inhibition is mediated by GABA causing a depolarization of primary afferent terminals (*Eccles* et al., 1963; *Schmidt,* 1971; *Curtis* and *Johnston,* 1974; *Krnjević*, 1974). Administration of GABA to cultured dorsal root ganglion (DRG) cells caused a marked and rapid depolarization, often producing action potentials (*Obata,* 1974; *Lawson* et al., 1976; *Hösli* et al., 1977, 1978). This depolarization was accompanied by an increase in membrane conductance. Testing the action of GABA upon repeated applications at short intervals, it was observed that the amplitude of the depolarization decreased progressively indicating receptor desensitization (*Hösli* et al., 1977, 1978). Desensitization to the depolarizing action of GABA was also seen in rat superior cervical ganglia (*Bowery* and *Brown,* 1974; *Adams* and *Brown,* 1975) and on DRG cells of the rat in situ (*Deschenes* et al., 1976). In contrast to the pronounced effects of GABA, glycine had no action on cultured DRG cells (*Ransom* and *Nelson,* 1975). Investigations on ionic mechanisms associated with the action of GABA on isolated sympathetic ganglia (*Adams* and *Brown,* 1975) and on DRG cells in vivo (*Deschenes* et al., 1976) and in tissue culture (*Hösli* et al., 1978) provide evidence that the GABA-induced depolarization is mainly dependent on chloride ions.

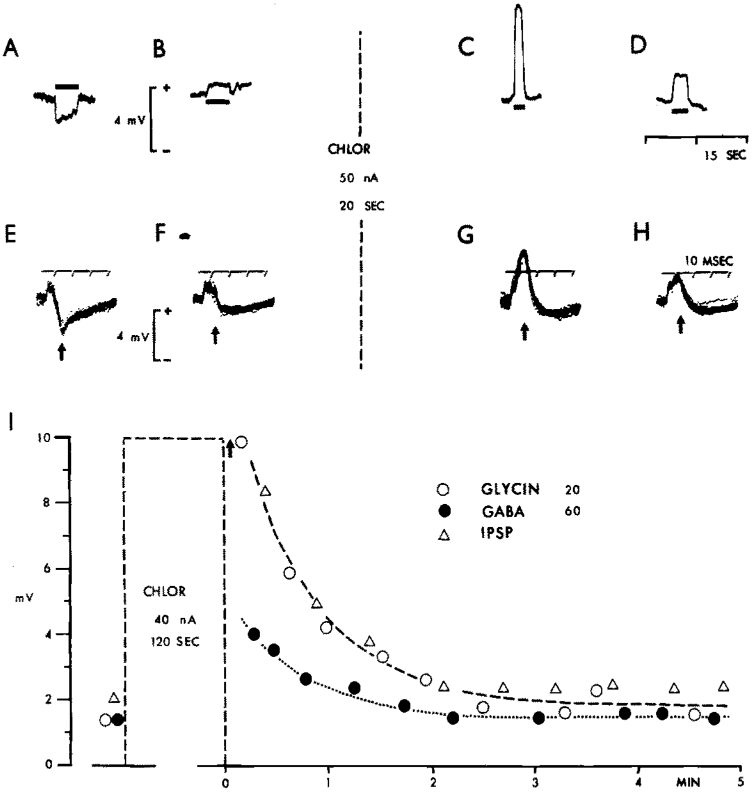
6.2. Effects of Excitatory Amino Acids

There is much evidence indicating that the acidic amino acids glutamate and aspartate may function as excitatory transmitters in many regions of the mammalian CNS (for refs. see *Johnson,* 1972; *Werman,* 1972; *Curtis* and *Johnston,* 1974; *Krnjević,* 1974). Microelectrophoretically administered glutamate and aspartate caused an increase of the firing frequency and a depolarization of the cell membrane of CNS neurones in situ (*Bernardi* et al., 1972; *Curtis* et al., 1972; *Curtis* and *Johnston,* 1974; *Krnjević,* 1974). Glutamate and aspartate applied microelectrophoretically (Fig. 8B) or added to the bathing fluid in concentrations of 10^{-3} to 10^{-5} M also caused a depolarization (Figs. 11A, B, 12, 13A) of spinal neurones in tissue culture, similar to that observed on spinal neurones in situ (*Hösli* et al., 1973a, c, 1976a; *Ransom* et al., 1975; *Ransom* and *Nelson,* 1975). This depolarization was often accompanied by a discharge of action potentials. As was also observed in the spinal cord in situ (*Bernardi* et al., 1972; *Curtis* et al., 1972), the depolarization by the excitatory amino acids was associated with a decrease in membrane resistance which was, however, much smaller than the conductance change produced by inhibitory amino acids (*Hösli* et al., 1973a, 1976a; *Ransom* and *Nelson,* 1975).

Using the same iontophoretic currents or the same concentration of the amino acids in the bathing fluid, there were often considerable differences in the amplitude of the depolarizations between individual neurones (*Hösli* et al., 1973a, 1976a; *Ransom* and *Nelson,* 1975). These differences of effects which were also observed on CNS neurones in situ (*Curtis* et al., 1960; *Duggan,* 1974; *Johnston* et al., 1974) could either be explained by a differential sensitivity of the neurones to the amino acids or by the fact that the sub-

▶

Fig. 10 A - L. Influence of intracellular chloride injection on glycine action and an IPSP. Unidentified motoneurone, resting potential -65 mV, KCl recording electrode. (A - D) Alterations of membrane potential in response to electrophoretically administered glycine (30 nA), indicated by *black bar.* (E - H) Postsynaptic potentials evoked by stimulation of sural nerve, *arrow* indicates the position of the peak of IPSP in E. A,E: 4 - 5 min after impalement; B,F: 90 s later; C,G: immediately after the intracellular injection of chloride ions, 50 nA for 20 s; D,H: 3 min later. Calibrations: 4 mV for A - H; time: 15 s for A - D, 10 ms for E - H. (I) Another series from the same neurone showing the influence of an intracellular injection (40 nA, 120 s) on depolarizing potentials (mV) produced by electrophoretically administered glycine (o, 20 nA, 3 s) and GABA (●, 60 nA, 4 s) and the depolarizing component (Δ) of the postsynaptic potential corresponding to the original hyperpolarization. *Arrow* indicates that this depolarization exceeded threshold for spike production. *Ordinate:* change in resting potential, mV. *Abscissa:* time in minutes; the chloride injection time is not to scale (*Curtis* et al., 1968). Action of glycine (Glyc $10^{-4}M$) on the membrane potential of two different spinal neurones in tissue culture. (K) In normal bathing fluid. (L) After removal of extracellular chloride ions (Cl^--free bathing solution). Duration of perfusion with glycine is indicated by *horizontal bar* above tracings. *Ordinate:* membrane potential in mV. Time: 10 s for A, 20 s for B (*Hösli* et al., 1973c)


A
B
4 mV
+
-
CHLOR
50 nA
20 SEC
C
D
15 SEC
E
F
4 mV
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8
6
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2
0
mV
CHLOR
40 nA
120 SEC
○ GLYCIN 20
● GABA 60
△ IPSP
0
1
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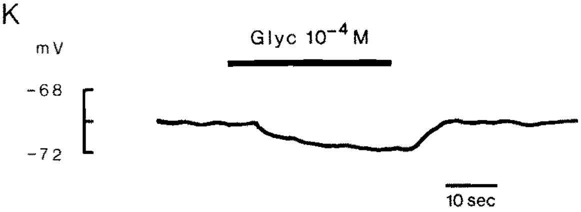
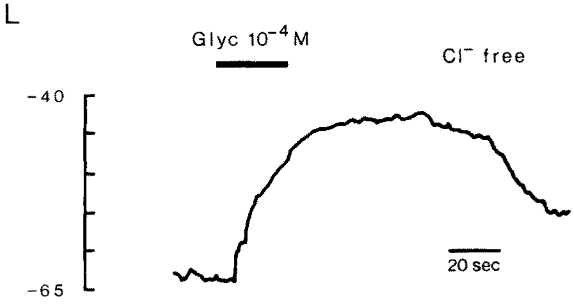
MIN
5


K
mV
Glyc 10^{-4} M
-68
-72
10 sec


L
Glyc 10^{-4} M
Cl⁻ free
-40
-65
20 sec

stances were hindered from reaching the neuronal membrane due to thin layers of astrocytic processes covering the surface of the cultures (*Guillery* et al., 1970; *Ransom* and *Nelson,* 1975; *Hösli* et al., 1975b, 1976a). However, when glutamate and aspartate were tested at various concentrations on the same cell, there was a clear dose-response relationship between the magnitude of effects and the concentration of the amino acids in the bathing fluid (Fig. 11A) (*Hösli* et al., 1973a, 1976a). Glutamate at a concentration of 10^{-4} M always caused a marked depolarization, whereas the amino acid was often ineffective (Fig. 11A) or produced only small depolarizations at a concentration of 10^{-6} M, indicating that 10^{-6} M might be the threshold concentration being similar to that described on snail neurones (*Gerschenfeld* and *Lasansky,* 1964). In contrast to the marked depolarizing action of glutamate on cultured spinal neurones, only slight effects, or none, were observed on DRG cells (*Obata,* 1974; *Ransom* and *Nelson,* 1975; *Ransom* et al., 1975; *Hösli* et al., 1977b).

No signs of desensitization to glutamate were detected after prolonged (Fig. 11B) or subsequent applications of the amino acid at short intervals (*Hösli* et al., 1973a, 1976a; *Ransom* and *Nelson,* 1975). These observations are also consistent with findings in the frog spinal cord (*Barker* and *Nicoll,* 1973) and in the mammalian CNS (*Curtis* et al., 1960; *Krnjević* and *Phillis,* 1963), where no desensitization to glutamate has been observed.

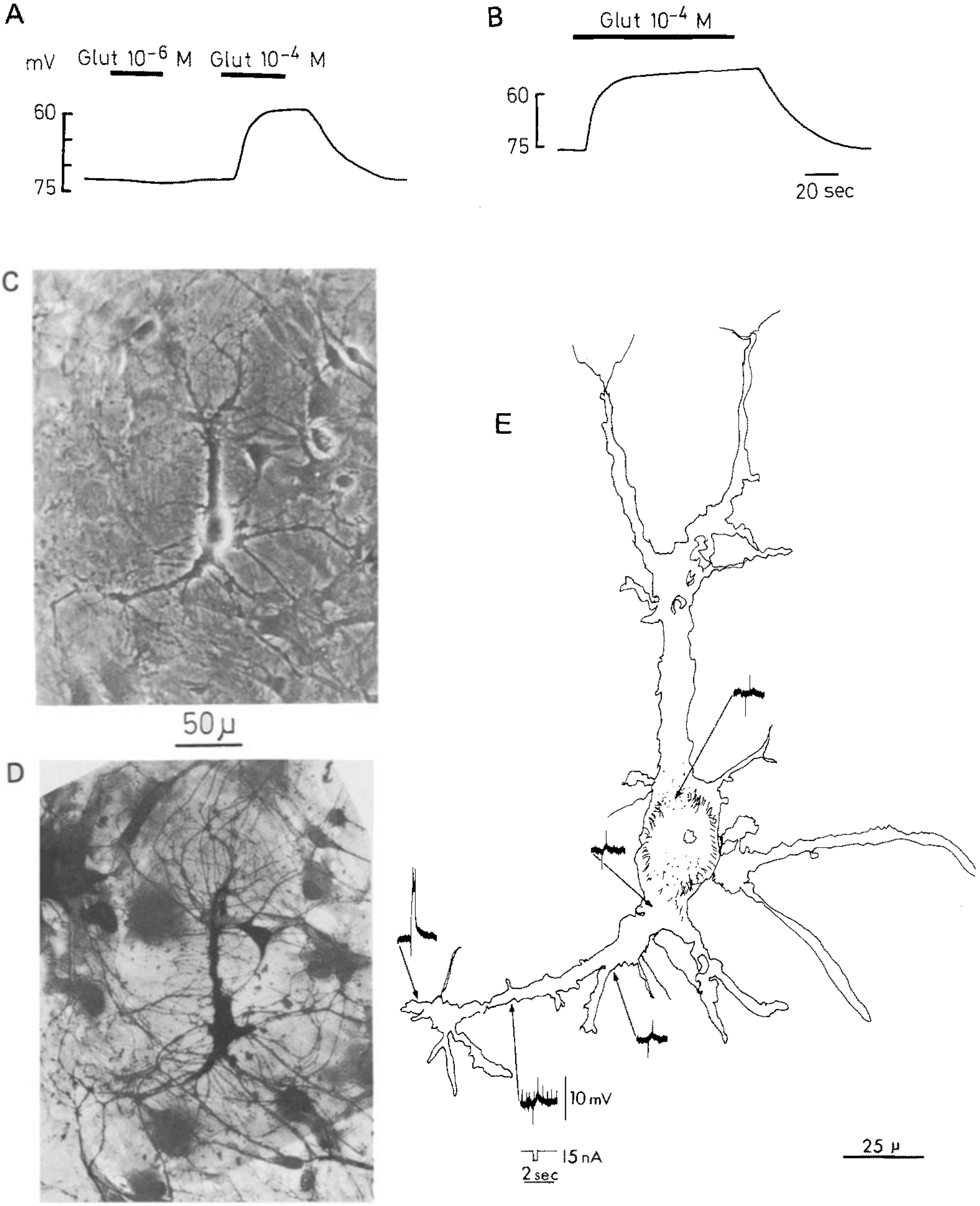
Electron-microscopic studies from our laboratory revealed that a great number of axosomatic and axodendritic synapses are found on neurones lying in the dense zones (explant and its margin) of spinal cord cultures, whereas neurones in the outgrowth zone have very few or no synaptic contacts (*Hösli* et al., 1975b). A comparison of the effects of glutamate and aspartate between neurones lying in the dense zones and those located in the outgrowth zone suggests that there is no difference in sensitivity between neurones making numerous synaptic contacts, or only few, or none. *Woodward* et al. (1971) have also observed that cerebellar Purkinje cells of newborn rats

Fig. 11. (A, B) Effect of glutamate (*Glut*) on the membrane potential of cultured human spinal neurones. (A) Glutamate at a concentration of 10^{-6} M (*Glut* 10^{-6} M) had no effect. Addition of flutamate at a concentration of 10^{-4} M (*Glut* 10^{-4} M) caused a depolarization of 15 mV. (B) Sustained depolarization by glutamate (*Glut* 10^{-4} M) applied for approximately 80 s. Duration of perfusion with glutamate is indicated by *bar* above tracings. *Ordinate:* membrane potential in mV. Time: 20 s. Culture: 14 days in vitro; fetus: 8 weeks in utero (*Hösli* et al., 1976a). (C - E) Spinal cord cell exhibiting a localized site of increased sensitivity to glutamate. Insets show a phase optics photomicrograph of the living cell (C) sketched in E and a subsequent bright-field photomicrograph of the same cell after silver staining (D). In E, penwriter records are shown of responses elicited by the glutamate test pulse at different areas of the cell. Glutamate pulse parameters are shown only once at the bottom of E. Note dramatic increase in response amplitude obtained when glutamate is applied to the tip of the cell process seen at *bottom-left* in E. Note also faster rise time at the "hot spot".
Lowermost voltage trace contains a number of vertical spikes that represent a burst of spontaneously occurring EPSPs (*Nelson,* 1975a)

▶

are sensitive to GABA, NA, cAMP, and glutamate before synaptic contacts have been formed. Furthermore, cultured neuroblastoma cells which are known to have no synapses were also affected by several neurotransmitters (*Peacock* and *Nelson,* 1973). These findings suggest that the neuronal membrane can exhibit chemosensitivity before synapses are formed.

Comparing the action of glutamate and aspartate between neurones of human and rat spinal cord cultures, it was observed that human spinal neu-



rones were usually more sensitive to the amino acids than rat neurones (*Hösli* et al., unpublished observations). This variation in sensitivity might be due to differences in species or in the ontogenetic development of the nervous tissue used. Since the cultures of human spinal cord were obtained from fetuses at a much earlier developmental stage (8-10 weeks in utero) than those of rats (18 days in utero or newborn), it is suggested that fetal cells may have a greater sensitivity to neurotransmitters than neonatal and adult neurones. *Woodward* et al. (1971) have also seen that cerebellar Purkinje cells of neonatal animals exhibited a much stronger response to iontophoretically applied neurotransmitters than neurones of adult animals.

The method of tissue culture offers an unique possibility for the study of different areas of a neurone in respect to responsiveness to transmitter substances and for the search for so-called "hot spots" on the neuronal membrane to a specific excitatory or inhibitory transmitter. After focal application of glutamate to different areas of cultured spinal neurones, it has been demonstrated that mainly the processes of the cells tested contained areas where the membrane seemed particularly sensitive to the amino acid (Fig. 11E) (*Nelson,* 1975a). In contrast, when spinal neurones were studied with regard to their sensitivity to the inhibitory transmitters glycine and GABA, it was found that the sensitivity was highest over the soma area (*Ransom* and *Nelson,* 1975). These findings are consistent with studies by *Eccles* (1957), suggesting that inhibitory synapses are mainly located on the soma, whereas the majority of excitatory synapses probably terminate on the dendritic tree of neurones.

Although there is little information on ionic mechanisms associated with the depolarization produced by glutamate and aspartate on neurones of the mammalian CNS, there is good evidence from studies on invertebrates indicating that sodium is the predominant ion responsible for the currents producing the excitatory junctional potential as well as the depolarization induced by glutamate (*Takeuchi* and *Onodera,* 1973; *Anwyl* and *Usherwood,* 1974). Investigations on ionic mechanisms underlying the action of glutamate and aspartate on cultured human and rat spinal neurones also suggest that sodium ions are mainly responsible for the amino acid induced depolarization (*Hösli* et al., 1973a, c, 1975b, 1976a). Thus, replacement of sodium ions by choline in the extracellular fluid reversibly abolished the depolarization produced by glutamate (Fig. 12) and aspartate, indicating an increased sodium conductance of the neuronal membrane (*Hösli* et al., 1973a, c, 1975b, 1976a). These findings are in agreement with observations made on spinal neurones in situ, indicating that the EPSP as well as the depolarization by glutamate may involve an increase in membrane permeability to sodium ions (*Eccles,* 1964; *Curtis* et al., 1972; *Barker* and *Nicoll,* 1973).

A number of electrophysiologic studies have shown that excitable cells still produce action potentials when sodium ions were replaced by lithium in the extracellular fluid (*Huxley* and *Stämpfli,* 1951; *Keynes* and *Swan,* 1959; *Armett* and *Ritchie,* 1963). In contrast, the depolarizing action of glutamate and aspartate on cultured spinal neurones was abolished when sodium ions were replaced by lithium in the bathing fluid (*Hösli* et al., 1976a), indicating that lithium cannot substitute for sodium ions for the amino acid depolarizations, as it can in the process of generating the action potential. These results are in agreement with studies on the crayfish neuromuscular junction by *Ozeki* and *Grundfest* (1967), demonstrating that the EPSP but not the action potential disappeared slowly after substituting lithium ions for sodium. Furthermore, tetrodotoxin (TTX) selectively blocked the action potentials but did not affect the EPSP and the glutamate-induced depolarization of spinal neurones in situ (*Curtis* et al., 1972; *Zieglgänsberger* and *Puil,* 1972; *Barker* and *Nicoll,* 1973). It is, therefore, concluded that although the action potential, the synaptic potential and the amino acid de-

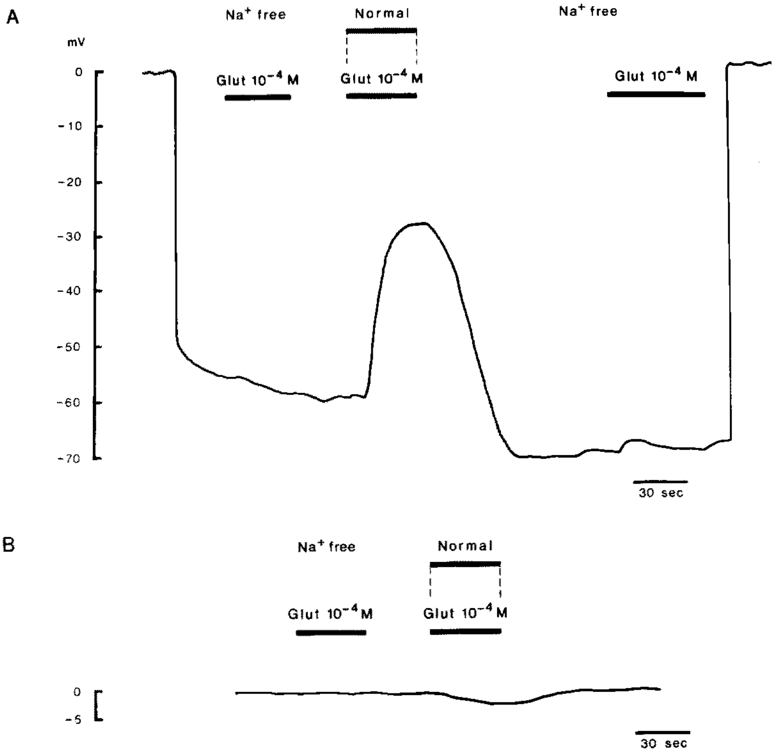


Fig. 12 A and B. Effects of removing external sodium ions on the response to glutamate (*Glut* 10^{-4} *M*) on a human spinal neurone in tissue culture (16 days in vitro, fetus 9 weeks in utero). Perfusion with sodium-free (Na^+-free) solution was started approximately 3 min before impalement of the cell by the microelectrode (1 *M* K acetate). (A) Effects of glutamate (10^{-4}*M*) on the membrane potential in sodium-free (Na^+-free) and in normal (137 m*M* Na^+) bathing solution. The progressive increase in membrane potential is probably due to a "sealing-in" of the recording electrode. (B) Effects of normal and sodium-free bathing solutions were tested after withdrawal of the recording electrode from the cell. *Ordinate:* membrane potential in mV. Time: 30 s (*Hösli* et al., 1973a)

polarizations are associated with an increased sodium permeability, the sodium channels activated during the generation of the action potential might be different from those activated during the synaptically and/or amino acid induced depolarizations (*Hösli* et al., 1976a).

6.3. Effects of Acetylcholine and Monoamines on Cultured Neurones and Neuroblastoma Cells

A great number of biochemical and electrophysiologic investigations provide much evidence that acetylcholine (ACh) and monoamines such as noradrenaline (NA), dopamine (DA), and serotonin (5-HT) may act as transmitter substances in the mammalian CNS (*Carlsson,* 1959; *Bradley,* 1968; *Curtis* and *Crawford,* 1969; *McLennan,* 1970; *Phillis,* 1970; *Krnjević*, 1974). After microelectrophoretic application of ACh to cultured neuroblastoma cells, various types of responses have been observed. Some neuroblastoma cells were depolarized, whereas other cells were hyperpolarized by ACh. Bi- and triphasic combinations of depolarizing and hyperpolarizing actions were also seen (*Harris* and *Dennis,* 1970; *Nelson* et al., 1971; *Peacock* and *Nelson,* 1973). All the responses to ACh were accompanied by a decrease in membrane resistance. ACh administered to cultured neurones of fetal rat cortex produced an initial depolarization followed by a hyperpolarization (*Godfrey* et al., 1975). However, *Bonkowsky* and *Dryden* (1976) observed only depolarizing effects of ACh on disaggregated brain cells in culture. These observations correlate well with investigations on neurones in the CNS in vivo, where excitatory and inhibitory effects of ACh have also been described (for ref. see *Krnjević*, 1974). Microelectrophoretically applied ACh produced a depolarization of cultured muscle cells, and this effect as well as the endplate potentials were blocked by curare (*Fischbach,* 1970; *Kano* and *Shimada,* 1971; *Fischbach* and *Cohen,* 1973). ACh also caused a depolarization and a discharge of action potentials of vegetative ganglion cells in tissue culture (*Obata,* 1974; *Ko* et al., 1976). Administration of tetrodotoxin blocked the action potential of cultured vegetative ganglion cells without affecting the membrane potential and the depolarizing action of ACh (*Obata,* 1974; *Ko* et al., 1976). In contrast, ACh had no effect on the membrane potential of cultured DRG cells (*Obata,* 1974).

After administration of monoamines to cultured neuroblastoma cells, it was found that DA hyperpolarized the cell membrane of about one-quarter of the cells tested, whereas NA and 5-HT had no effect (*Peacock* and *Nelson,* 1973). Application of NA to cultured neurones of fetal rat cortex caused a hyperpolarization (*Godfrey* et al., 1975), whereas NA, DA, and 5-HT had only depolarizing actions on disaggregated brain cells in tissue culture (*Bonkowsky* and *Dryden,* 1976). Monoamines usually had depressant effects on the glutamate-induced firing of cultured tuberal hypothalamic neurones (*Geller,* 1976). Depression by NA of the discharge rate of spontaneously firing Purkinje cells has been described in the cerebellum in vivo (*Hoffer* et al., 1971; *Siggins* et al., 1976), in cerebellar transplants (*Hoffer* et al., 1975), and in cerebellum in tissue culture (*Gähwiler,* 1975b), indicating that Purkinje cells in vitro are susceptible to NA similar to those in vivo.

7. Action of Neurotransmitters on Cultured Glial Cells

Although amino acid transmitters are taken up to a great extent by cultured glial cells (*Hösli* et al., 1975b; *Hösli* and *Hösli,* 1976a), electrophysiologic studies have shown that glycine, glutamate, and aspartate usually had no action on the membrane potential of glial cells in human and rat CNS tissue culture (Fig. 13B, C) (*Wardell,* 1966; *Hösli* et al., 1975b, 1976b). GABA often had no effect, although a slight depolarization was observed on some astrocytes (Fig. 13C) (*Hösli* et al., 1976b). Recent investigations on satellite glial cells of cultured rat DRG have, however, shown that GABA depolarized the membrane of almost all glial cells studied (*Hösli* et al., 1977). Microelectrophoretically applied GABA also caused a depolarization or had no effect on glial cells in the cortex of the cat in situ (*Krnjević* and *Schwartz,* 1967). No change in membrane resistance was observed, even when GABA had a clear depolarizing action (*Krnjević* and *Schwartz,* 1967). Monoamine

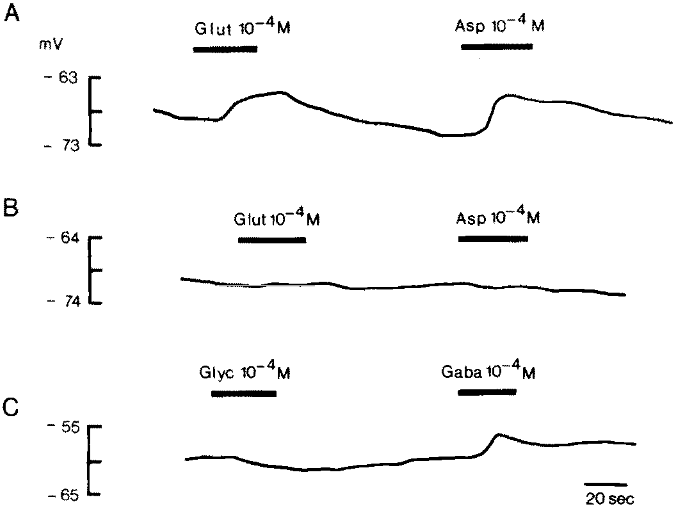


Fig. 13. (A, B) Effects of glutamate (Glut $10^{-4}M$) and aspartate (Asp $10^{-4}M$) on the membrane potential of a neurone (A) and a glial cell (B) in the same culture (rat spinal cord, 16 days in vitro). (C) Action of glycine (Glyc $10^{-4}M$) and GABA ($10^{-4}M$) on a glial cell of another rat spinal cord culture (20 days in vitro). Duration of perfusion with the amino acids is indicated by *horizontal bars* above tracings. *Ordinate:* membrane potential in mV, *bar* represents 20 s (*Hösli* et al., 1976b)

transmitters such as NA and 5-HT also had no effects on glial cells in tissue culture (*Wardell,* 1966) and in situ (*Krnjević* and *Schwartz,* 1967).

In contrast to the results obtained with amino acid and monoamine transmitters, ACh was found to have depolarizing and hyperpolarizing actions on glial cells. Microelectrophoretic application of ACh to glial cells in the cortex of the cat in situ caused a depolarization of one-half the cells tested, whereas the membrane potential of the other glial cells remained unaffected (*Krnjević* and *Schwartz,* 1967). There was an increase in membrane resistance during the depolarization by ACh (*Krnjević* and *Schwartz,* 1967). In studies on cultured glioma cells, it was observed that ACh produced a hyperpolarization which could be blocked by atropine but not by D-tubocurarine suggesting the existence of a muscarinic ACh receptor (*Hamprecht* et al., 1976). In cerebellar cultures, however, microelectrophoretically administered ACh had no action on the membrane potential of glial cells (*Wardell,* 1966).

From the observations that the membrane potential of most glial cells is not affected by monoamine and certain amino acid transmitters, it is concluded that, unlike neurones, glial cells may not possess receptors, which after combining with these transmitter substances, alter the membrane permeability for specific ions, thus producing changes in membrane potentials (*Hösli* et al., 1976b).

8. Summary and Conclusions

This review is mainly concerned with studies on the action and uptake of putative neurotransmitters in cultured CNS tissue. Some investigations on the presence and ontogenetic development of enzymes associated with neurotransmitters have also been included. The method of tissue culture has proved to be an exellent tool to study the cellular and fine-structural localization of the uptake of neurotransmitters using autoradiographic techniques. The amino acid transmitters GABA, glycine, glutamate and aspartate were found to be taken up not only by a great number of neurones of spinal cord, brain stem, and cerebellar cultures but to a great extent also by glial cells, suggesting that glial elements might also play a role in the inactivation of amino acid transmitters. In contrast, uptake of monoamine transmitters such as NA, DA, and 5-HT was only observed in nerve fibers and in a few neurones but not into glial cells.

Tissue culture techniques also provide a useful model to investigate the action of neurotransmitters on single neurones and glial cells of the mammalian CNS by means of microelectrodes under direct visual control and to study ionic mechanisms associated with transmitter actions by altering the composition of the extracellular fluid.

As was also observed in the mammalian CNS in vivo, glycine, GABA, and taurine caused a hyperpolarization, whereas glutamate and aspartate depolarized the neuronal membrane, both effects being accompanied by an increase in membrane conductance. From these results, it is concluded that cultured neurones possess receptors for the amino acids which are similar to those of neurones in the CNS in situ. In contrast, amino acid transmitters usually had no effects on the membrane potential of cultured glial cells.

The depolarizing actions of the excitatory transmitters glutamate and aspartate were reversibly reduced or abolished when sodium ions were replaced by choline or lithium in the extracellular fluid, suggesting that the depolarization of these amino acids is mainly dependent on an increased sodium permeability. Removal of chloride ions from the bathing solution reversed the hyperpolarization produced by glycine to a depolarization, indicating that this amino acid alters the permeability of the neuronal membrane for chloride ions as it does on neurones of the CNS in situ.

From these studies, it is concluded that the method of tissue culture is a valuable tool to study the cellular localization of the uptake of neurotransmitters as well as to investigate the action and associated ionic mechanisms of transmitter substances on neurones and glial cells of the mammalian and particularly of the human CNS.

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