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Immobilized and Insolubilized Drugs, Hormones, and Neurotransmitters: Properties, Mechanisms of Action and Applications*

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I. Introduction

THE immobilization or covalent bonding of drugs,† hormones, and neurotransmitters to soluble and insoluble supporting matrices is a technique that has found application to diverse endeavors ranging from elucidation of the sites and mechanisms of hormone action to drug receptor isolation and clinical therapeutics. Because of this wide ranging utility and the importance of the basic science questions involved, a major portion of this review will be devoted to the examination of the hypothesis that drugs and hormones can evoke specific physiological and pharmacological responses while remaining covalently coupled to a supporting matrix. The remainder of this article will examine some of the applications of immobilized drugs to understanding the sites and mechanism of drug and hormone action and the role for immobilized drugs as therapeutic agents.

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† The term "drugs" is used loosely here to mean substances of exogenous origin including enzymes applied for pharmacological or therapeutic purposes.

A. History of Immobilization

One of the first uses of small molecules bound to a macromolecular support can be traced to the 1920s when reactions with haptenic groups were elucidated. Such studies involving, e.g., *p*-aminobenzene-arsenate diazotized to various proteins were used in determining antibody specificities (199). Lerman and his coworkers in 1951 developed the technique, now referred to as "affinity chromatography," when they covalently linked an antigen via an azo bond to a cellulose matrix for the purification of antibodies (43, 208). In 1953 Lerman applied the new technique to enzyme purification when he utilized a series of *p*-azophenyl derivatives linked to a cellulose matrix for the affinity isolation of tyrosinase (209).

In 1954 Jatzkewitz bound mescaline to a synthetic copolymer of vinylpyrrolidone and acrylic acid. Mescaline, which was covalently coupled to the copolymer via

a dipeptide side arm, developed resistance to elimination as a result of its attachment to the copolymer (165, 166).

The area of immobilized enzymes can possibly trace its beginnings to 1916 when Nelson and Griffin reported on the adsorption of invertase on charcoal and alumina with retention of its enzymatic activity (242). But it was not until the work of Katchalski and coworkers in the 1960s that enzymes were covalently immobilized (124).

In 1962 Arakawa et al. applied immobilization techniques to the pharmacology of angiotensin, when these investigators immobilized angiotensin on a synthetic polymer of poly (O-acetylserine) and concluded from biological tests that angiotensin exerted its action on the cell membrane of target cells (11).

Schimmer, Sato, and coworkers in 1968 were the first to apply the techniques of insolubilization of small molecules on Sepharose beads to a biological system when they reported that adrenocorticotropic hormone (ACTH) retained biological activity while covalently bound to the Sepharose beads via an azo linkage. It was argued that ACTH by nature of its being bound covalently to particles larger than cells must be exerting its action on cells by a direct interaction with membrane receptors for ACTH (311).

Cuatrecasas in 1969 drew similar conclusions for the mechanism of action of insulin on isolated fat cells by using insulin covalently attached to Sepharose beads (62). Richardson and Beaulnes (296) extended the studies of Arakawa et al. (11) by coupling angiotensin to soluble enzymes and reported that the complexes retained biological activity on the outside of cells. In studies with catecholamines covalently bound to glass beads, Venter, Kaplan and co-workers (375, 380, 381) applied immobilized drugs to various cardiac preparations in an attempt to study the sites and mechanism of action of these hormones and neurotransmitters. About the same time, Johnson et al. (167) presented data suggesting that glucagon-Sepharose and norepinephrine bound via an amide linkage to Sepharose possessed biological activity when tested on isolated fat cells.

In addition to these studies, designed to locate the cellular site and/or mechanism of hormone action, were studies with chemotherapy in mind such as those by Moolten et al. (238, 239) who used diphtheria toxin conjugated to an antibody that was directed to a cell surface antigen. During and since these studies, a wide variety of reports have appeared concerning the biological activity of "immobilized" drugs, hormones, and neurotransmitters (tables 1 to 3).

II. Can Drugs, Hormones, and Neurotransmitters Retain Biological Activity While Covalently Coupled to Supporting Matrices?

A. Definition of Biological Activity of Immobilized Drugs

Understanding the mechanism of action of immobilized drugs is intertwined with basic receptor theory and receptor mechanisms. Our understanding of immobilized drugs is limited to a certain extent by our knowledge of receptors. The converse is also true in that our knowledge of drug receptors is, and has been, somewhat dependent on immobilized drug preparations. Immobilized drug and hormone preparations have been used to localize receptors to specific parts of cells (8–11, 21, 24, 44, 62, 98, 113, 125, 139, 163, 164, 167, 180, 197, 224, 228, 229, 249, 251, 252, 256, 279, 295, 296, 298, 308, 311, 316, 319, 334, 336, 341, 347, 351, 361, 369, 375, 380, 381, 383, 395, 398, 405), to isolate and purify soluble and membrane-bound receptors (table 2), to understand structure activity relationships of drugs on specific receptors (11, 34, 44, 62, 142, 176, 224, 226, 229, 251, 252, 296, 311, 320, 332, 334, 341, 369, 373, 375, 378, 380, 384, 395, 397), and to understand the mechanism of receptor expression (16, 21, 34, 149, 156, 226, 251, 252, 320, 321, 334, 341, 351, 370, 371, 375, 380, 381, 383).

The origin of the receptor concept is generally attributed to Ehrlich (99) and Langley (203) in the early part of this century. However, evidence localizing receptors for many hormones and neurotransmitters to the plasma membranes of cells and knowledge of the chemical nature of receptors comes only from the last decade. Throughout this century substantial amounts of information have been compiled with regard to certain neurotransmitter and hormone receptors. Much of this information has been and is being utilized to characterize receptors in conjunction with direct binding studies. Recent reviews have outlined specific criteria that should be met in attempting to correlate direct, radiolabeled ligand binding to receptor function (71, 72, 69, 70, 78, 75, 146, 170, 171, 355). Many of these criteria, including similarities in structure activity relationships for binding and biological activity and the notion of stereospecificity, which apply to liggand-receptor binding studies can also be directly applied to immobilized drug studies (table 4).

1. Receptor binding. Biological activity of immobilized drugs can be defined in terms of receptor theory. An important prerequisite for most drug and hormone action is that a ligand bind to its physiologically relevant receptor site. Therefore, one definable form of biological activity of an immobilized drug is a specific receptor interaction between a covalently immobilized drug molecule and its target receptor.

2. Effector responses. As a consequence of a drug receptor interaction, a physiological response may be evoked if the drug is acting as an agonist. However if the drug is merely occupying the receptor, i.e., the drug is acting as a pharmacological antagonist, it is possible that no physiological response may occur.

While these concepts are the fundamentals of pharmacology, the existence of only one type of response, namely an immobilized drug-receptor interaction, does not necessarily prove that receptor activation can occur by the immobilized ligand. Conversely, the production of a physiological response by an "immobilized drug preparation" does not necessarily indicate that an interaction

TABLE 1

Solid phase immobilized drugs, hormones, and neurotransmitters

Matrix	Immobilized Drug	References and Purpose of Study
Sepharose	ACTH	311,[6] 369,[2] 300[1]
	6-Aminopenicillanic acid	25,[1] 26,[1] 195,[1] 341,[6] 343,[1] 391[1]
	Antibody	14[2]
	Adenosine triphosphate	147[1]
	β-Receptor antagonists	45,[3] 44,[6,9] 342,[3] 363,[1] 362,[3] 406[9]
	Biotin	79,[1] 143[1]
	Cardiac glycosides	252,[6] 409[1]
	Catecholamines	44,[5] 204,[6] 224,[2] 228,[2] 229,[2] 363,[1,3] 369,[2] 365,[9] 395[2]
	Concanavalin A	4,[3] 8,[6] 9,[6] 31,[1] 98,[2] 125,[8] 256,[6] 300,[1] 345[3]
	Cortisol	300[1]
	Deoxycorticosterone	103,[3] 213,[1] 214[1]
	Diptheria toxin	369[2]
	DNP-albumin	82[2]
	Estradiol	328,[3] 329,[3] 385[3]
	Gangliosides	78[3]
	Glucagon	167,[6] 197[3]
	Heparin	235[3]
	Histamine	395,[2,6] 320,[2,6] 319,[2,6] 229,[2,6] 225,[2,6] 227,[2,6] 298,[6] 228,[2,6] 224,[6] 34[2,6]
	Human chorionic gonadatropin	95,[3] 96,[3] 97[3]
	Human growth hormone	29,[9] 134,[1] 318,[6] 327,[3] 326[3]
	5-Hydroxytryptamine	325,[3] 407[9]
	Insulin	68,[5] 62,[5] 143,[5] 65,[3] 160,[3] 1,[1] 24,[6] 61,[1] 66,[3] 74,[3] 77,[3] 81,[6,9] 137,[3] 190,[9] 159,[3] 181,[9] 200,[3] 361,[6] 249,[6] 248,[6] 250,[9] 336,[2,6] 397,[5,9] 180[2,6]
	Interferon	10[6]
	Lectins	126[2,6]
	Leuteinizing hormone	96[6]
	Methotrexate	182–185[1]
	Morphine (analogs)	330,[1] 331,[3] 332[1]
	Nerve growth factor	113[6]
	Placental lactogen	386[9]
	Prolactin	361,[6] 386[9]
	Prostaglandins	225,[2] 333[6]
	Pyridoxamine phosphate	232,[1] 233[1]
	Quaternary ammonium ions	23,[3] 253,[3] 392,[3] 314,[3] 334,[3] 294,[3] 178,[3] 293,[3] 177,[3] 47[3]
	Renin substrate	132,[1] 287[1]
	Thyroid hormone	265,[1] 369[2]
	Thyrotropic hormone	352[3]
Glass beads	Antibody	141[1]
	Cardiac glycosides	370[6,9]
	Catalase	382[7]
	Catecholamines	21,[6] 16,[6] 369,[2] 371,[6,9] 373,[5,9] 375,[6] 378,[3] 379,[9] 380,[6,9] 381,[6] 399,[9] 406,[9] 407[9]
	Glycosphingolipid	405[5,6]
	Growth hormone	28[1]
	5-Hydroxytryptamine	407[9]

TABLE 1—*Continued*

Matrix	Immobilized Drug	References and Purpose of Study
	Propranolol	380[6]
	Uricase	382[7]
Glass plates	Glycolipids	405[6,5,2]
Polyacrylamide	N-Acetylglucosamine	316[2]
	β-Lactoside haptens	139,[2] 360,[2] 398[2]
	Bovine serum albumin	157[2]
	Human γ-globulin	14[2]
	Human serum albumin	157[2]
Sephadex	Antibody	313[2]
	Galactose	51[2,6]
Cellulose	Estradiol	385[3]
	Bovine serum albumin	43[1]
	p-Azobenzenearsonate	208[1]
	Azophenyl dyes	209[1]
	DNA	175[3]
	Polycytidylate	279[6]
Polystyrene	Estradiol	385[3]
Nylon	Concanavalin A	98[2]
	Phenylalanine ammonialyase	6[7]
	Bovine serum albumin	98[2]
	DNP-bovine serum albumin	98[2]
Polyvinyl chloride tubes	Glucose oxidase	168[7]
	Glucose peroxidase	168[7]
	Urokinase	168[7]
Plastic Petri dishes	Concanavalin A	8,[2] 98[2]

[1] Affinity chromatography.
[2] Affinity isolation of cells.
[3] Affinity isolation of receptors.
[4] Affinity isolation of enzymes.
[5] Structure activity relationships.
[6] Elucidation of sites and mechanisms of drug action.
[7] Enzyme therapy.
[8] Targeting carriers to specific sites.
[9] Questioning immobilized drug data.

has occurred between the immobilized ligand and the receptor.

B. Can Drugs, Hormones, and Neurotransmitters Retain Biological Activity While Covalently Coupled to a Solid Support, e.g., Sepharose or Glass Beads?

This question is subtly different from question II outlined above in that it defines a specific subset of immobilized drugs, those immobilized on solid supports (table 1).

Although the concept of drugs acting while covalently bound appears widely accepted, the exact mechanism of "immobilized drug" action is by no means clear-cut and varies dramatically from system to system.

TABLE 2

Affinity isolation of receptors and cells with immobilized drugs and hormones

Receptor, or Cell System	Matrix	Ligand	Reference
Nicotine cholinergic receptor	Agarose	Quaternary ammonium ions	253, 23, 392, 314, 334, 294, 177, 178, 293, 47
	Agarose	Naga Naga α-neurotoxin	33, 48, 87, 100, 101, 135, 174, 179, 188, 255, 264, 292
	Agarose	Erabu-toxin b	230
	Agarose	Fluxedil	231
Enriched membranes	Poly (ethylene oxide)	Quaternary ammonium ions	108, 109
Liver cell membranes	Agarose	Glucagon	197
Lymphocytes	Acrylamide	β-Lactoside haptens	34, 320, 228, 229
		Histamine	
	Polyacrylamide	Azophenyl-β-lactoside	360, 398
	Polymethylmethacrylic	Bovine serum albumin	390
	Plastic beads	Human γ-globulin	14, 82
	Agarose	Norepinephrine	229
	Sephadex	Antibody	313
	Petri dishes	Mouse fibroblasts	396
Corticosteroid binding globulin	Agarose	Cortisol hemisuccinate	300
Aldosterone binding protein	Agarose	Deoxycorticosterone	213, 214
Thyrotropin receptor	Agarose	Thyrotropic hormone (TSH)	352
		Methotrexate	182–185
Dihydrofolic reductase	Agarose		
β-Adrenergic receptor	Agarose	Anti-β-receptor	
		Monoclonal antibodies	112, 376, 377
	Agarose	Alprenolol	45, 362
	Agarose	Isoproterenol	342 Venter*
	Agarose	Practolol	342, Venter*
β-Adrenergic receptor containing membranes	Glass	Isoproterenol	378
	Dextran	Isoproterenol	Venter*
Serotonin binding protein	Agarose	Serotonin	325
Penicillin binding protein	Agarose	6-Aminopenicillanic acid	25, 26, 341, 343, 404
Thryoxine binding globulin (TBG)			265
Insulin receptors	Agarose	Insulin	200, 160, 68, 66, 65, 74, 77, 137, 159
Insulin antibodies	Agarose	Insulin	61
Estradiol receptors		Estradiol	385
	Agarose	Heparin	328, 329
Estradiol isomerase	Polyethylene oxide	Estradiol	150
Lutenizing hormone receptor	Agarose	Lutenizing hormone	95
Chorionic gonadotropin receptor	Agarose	Lutenizing hormone and chorionic gonadotropin	95
Prolactin receptors	Agarose	Growth Hormone	326
Opiate receptors	Glass	Morphine	330, 331
	Agarose	Morphine	331, 332
Lectin receptors	Agarose	Concanavalin A	4, 345
Hepatocytes	Polyacrylamide	N-Acetyl glucosamine	316
	Glass	Glycolipid	405
Leukocytes	Agarose	Histamine	224, 228, 395
	Agarose	Norepinephrine	395
C6 glial cells	Agarose	Isoproterenol	369
	Glass	Isoproterenol	369
Adrenal tumor cells	Agarose	ACTH	369
Pituitary tumor cells	Agarose	Thyroid hormone (T_3)	369
HeLa cells	Agarose	Diptheria toxin	369
Fat cells	Agarose	Insulin	180, 336
Glucocorticoid receptor	Dextran-polyethylene		
	Glycol	DNA	2
	Agarose	Deoxycorticosterone	103
3T3SV Cells	Sephadex	Galactose	51

* Unpublished data.

The simplistic nature of the concept of coupling a drug to a large polymer or solid particle and testing the complex for biological activity is marred to a certain extent by the limits of the chemical stability of the bonds linking the drug to the matrix and of the supporting matrix itself. The concept in practice is also limited in some instances by the existence of noncovalently attached molecules that may be adsorbed to and subsequently released from

TABLE 3
Polymeric immobilized drugs

Drug	Carrier	Reference
ACTH	Biotin-avidin	144
Adriamycin	Immunogloblins	154, 155
	DNA	359
N-Acetylhomocysteine	Dextran	373
Albumin	Poly-L-lysine	323
Alprenolol	Dextran	278
	Bovine serum albumin	112
N-(4-aminobenzenesulfonyl)-N'-butylurea	Methacrylamide polymers	245
Amphetamine	Dextran	236
Ampicillin	Dextran	169
Angiotensin	Horseradish	
	Peroxidase	296
	Cytochrome *c*	296
	Poly-D,L-alanine	295
	Poly-O-acetyl serine	11
Aniline	Dextran	273
Cardiac glycosides	Albumin	42, 251, 334
	Myoglobin	334
Cadmium	Ferritin	307
Chlorambucil	Poly(N-vinylpyrrolidone-co-vinylamin)	111
	Immunoglobulins	305, 110, 119
Cyclazocine	Lactic acid polymer	217
Daunomycin	Immunoglobulins	153, 154, 211
	DNA	57
	Dextran	20
Daunorobicin	Polyglutamate	155
	Carboxymethylcellulose	155
	Carboxymethyldextran	155
	Alginic acid	155
	DNA	57, 86, 357
Doxorobicin	DNA	86, 317
Diazonium ions and dyes	Dextran	271
Diptheria toxin	Immunoglobulin	354, 403, 301, 216, 238, 239, 266
Diptheria toxin A-chain	Epidermal growth factor	46
	B-subunit of human chorionic gonadotropin	247
	Insulin	234
	Monophosphopentamannose	402
	Lectins	366
	Monoclonal antibody	28, 122
	Concanavalin A	121, 400
DNA	Dextran-PEG	2
Ephedrine	N-Vinylpyrrolidone	193
Epinephrine	Hydroxypropylglutamine-*p*-aminophenylalanine copolymer	85
Epidermal growth factor	Diptheria toxin	46
Estradiol	Dextran	151
	Polyethylene oxide	150
Ferritin	Avidin-biotin	138
Fluorescein isothiocyanate	α-Bungarotoxin	346
Ganglioside	Lysine-alanine copolymer	78
Gelonin	Concanavalin A	339
Glucose oxidase	Antibodies	267
Hemoglobin	Dextran	350
Histamine	Alanine-tyrosine copolymer	
	Rabbit serum albumin	226
Horseradish peroxidase	Poly-L-lysine	307, 323
Hoechst 33258	Dextran	243
Insulin	N-(2-hydroxypropyl)-methacrylamide and N-methacryloldiglycyl *p*-nitrophenyl ester copolymer	55
	Poly-N-vinylpyrrolidone	189, 337
	Biotin-avidin	143, 144, 220
	Acrylic acid polymers	22
	Dextran	12, 169, 347, 351
Radioactive iodine	Immunoglobulins	222
	$(Fab')_2$ fragments	18, 186, 187
Isoproterenol	Hydroxypropylglutamine-*p*-aminophenyl alanine-copolymer	383, 384, 199, 371
Lactoperoxidase	Antibodies	350
Lysozyme	Asialoglycoproteins	299
Mercury	Dextran	152, 19, 271, 273
Methotrexate	Divinylether-maleic-anhydride copolymer	117, 289
	Poly-L-lysine	117, 288, 306, 307, 324
	Polyethyleneimine	117, 289
	Carboxymethylcellulose	117, 289
	Poly (D-lysine)	324
	IgG	54
	α-Chymotrypsinogen	54, 52, 53
	Bovine serum albumin	52, 53, 54
	Dextran	54
Mescaline	Polyvinylpyrrolidone	165, 166
NAD(H)	Dextran	241
Naloxone	Polymeric hydrazides	15, 263
Nitrogen mustard	Fibrinogen	387
	Albumin	387
Nicotinic acid	Starch	290, 291

TABLE 3—*Continued*

Drug	Carrier	Reference
Novacaine	Dextran	236
Nucleic acids	Vinylpolymers	270, 276, 349
Phenylenediamine mustard	Dextran	302
	Polyglutamic acid	303
Phenylephedrine	N-Vinylpyrrolidone	193
Penicillin	N-Vinylpyrrolidone polymers	
Propranolol	Avidin-biotin	223
Proflavine	Dextran	243, 244
Retinal	Dextran	277
Ricin A-chain	IgM	247, 28
	Immunoglobulins	196
	Epidermal growth factor	145
Streptomycin B	Polymeric hydrazides	145
Triethylammonium	Polyethylene oxide	108, 109
Triton X-100	Inulin	274
	Dextran	274
	Amylose	274
	Cellulose	274

TABLE 4

Criteria to be met in attempting to demonstrate biological activity of an immobilized drug preparation

1. The drug or hormone should be covalently linked to the supporting matrix in a manner consistent with the structural activity relationship known for the parent compound. The drug to be coupled at the lowest ratio possible between drug and support.
2. Stereospecificity observed for the parent compound should be considered in the immobilized drug preparation.
3. Direct chemical interactions between the covalently immobilized drug and the target receptor should be demonstrated.
4. Biological responses consistent with specific receptor interactions should be demonstrated.
5. With soluble, immobilized drug preparations, e.g., polymeric drugs, the law of mass action should be obeyed in terms of receptor interactions.
6. Responses consistent with the diffusion properties of the macromolecular supports should be observed where applicable.
7. The amount of drug released from the matrix must be below that which would produce a biological response.

the matrix. Because of these and other limitations, controversies developed over the assignment of biological activity to the covalently immobilized form of drugs in various "immobilized drug" preparations (39, 81, 181, 190, 379, 406, 407).

Three principal mechanisms of action are proposed in figure 1 for immobilized drugs acting on biological systems. In the first mechanism (mechanism A), the supporting matrix to which a drug is chemically attached acts simply as a depot or source of soluble drug by releasing the drug into solution. The drug does not elicit a biological response unless a threshold concentration for that particular compound in solution is exceeded. The threshold level is the molar concentration of soluble drug that is necessary to elicit a biological response from the effector tissue. Included under mechanism A are any forms of gross drug leakage regardless of whether the released material was originally covalently bound or only adsorbed to the matrix. While such depot effects are striven for in the pharmaceutics of many compounds (15, 36, 52–54, 84, 88–90, 93, 111, 128, 129, 161, 165, 166, 172, 191, 192, 201, 202, 260), this cannot be construed as biological activity of immobilized drugs.

The second possible mechanism (mechanism B, fig. 1) represents a special situation in which previously covalently coupled or adsorbed drug is released at extremely limited rates, such that the free drug action is essentially confined to a microvolume created at a matrix-tissue or matrix-cell interface. The active species of drug, although

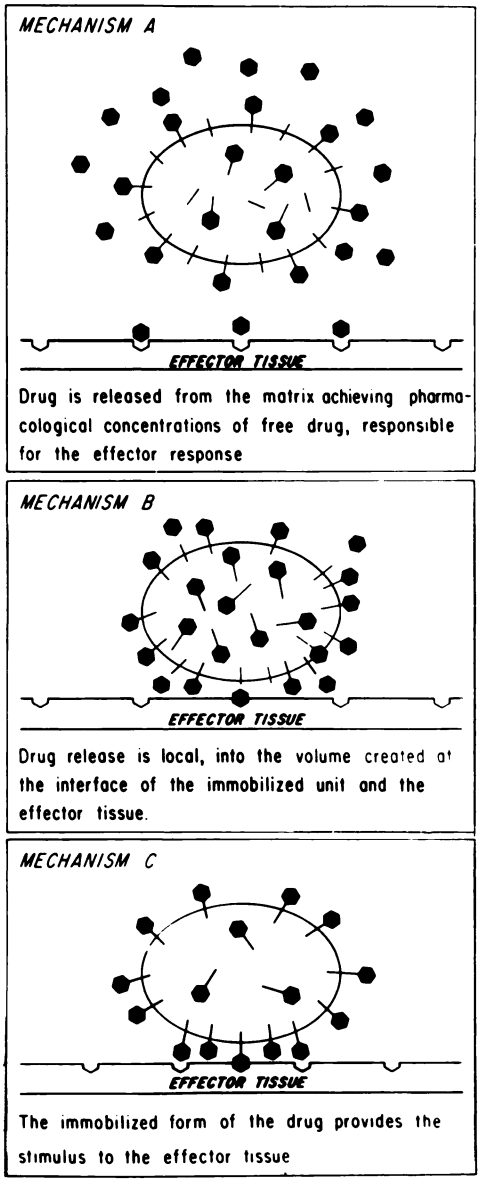

FIG. 1. Proposed mechanisms for immobilized drug action. See text for further details.

not coupled to the matrix, is located to the same volume or nearly the same volume as that occupied by the covalently coupled form of the drug. In the third possibility (mechanism C, fig. 1), the interaction of the covalently coupled form of the drug with specific cell membrane receptors is the only stimulus for the biological response. Obviously, various combinations of these mechanisms could theoretically exist. However, if experiments are performed under conditions where mechanism A operates it should be equally obvious that mechanism B and C would go undetected. Therefore, elimination of the mechanism A condition has been a principal experimental goal in working with immobilized drugs.

1. Chemistry of immobilization and immobilization techniques. The synthetic aspects of the chemistry of drug immobilization are substantially simplified over general organic chemistry by the ease of end product purification. For example, in the coupling of small molecules to solid phase matrices such as glass or Sepharose beads, excess reactants from intermediate coupling steps are simply washed away while retaining the derivatized beads in a Buchner funnel. In addition to the more obvious benefits of this simple chemistry, the final coupling of generally unstable biological compounds can occur under very mild conditions. Such "mild" reaction conditions enhance the chances of retaining biologically active molecules and limiting side reactions.

The chemistry of coupling to Sepharose, glass, and polyacrylamide beads has been the subject of many detailed articles and reviews (40, 56, 63, 64, 67, 73, 76, 80, 144, 162, 194, 215, 240, 262, 332, 374, 397). With the solid supports, side chains of desired length and composition can be easily attached, ending with the desired functional groups such as an amino, carboxyl, arylamine, hydroxyl, etc. Coupling reactions to soluble polymer complexes are in general only moderately more difficult than coupling reactions to solid supports. Due to the relatively simple chemistry involved in immobilizing drugs, the most important question will concern the method of coupling that is likely to produce a conjugate potentially consistent with the structure activity relationships for the drug in question (section IV). Some proposed structures for immobilized drugs are illustrated in figure 2.

2. The effects of synthesis and washing procedures on solid phase drug leakage rates. The synthesis and preparation of "immobilized" drugs can determine to certain extents the mechanisms of action of these complexes. The synthesis of drugs covalently bound to glass or Sepharose beads involves the placement of chemically activated glass or Sepharose particles into high concentrations of the drug to be covalently coupled. During chemical coupling a certain percentage of drug molecules become covalently attached to the reactive groups on the solid support, leaving a large number of noncoupled molecules both free in solution and entrapped in or adsorbed to the surface of the solid support. In order to assess the biological activity of the covalently coupled drug, the adsorbed drug molecules must be removed by adequate washing of the preparation. The solvent should be one in which the drug is highly soluble and which does not compromise the stability of the matrix. For example, due to the high acid solubility of catecholamines and the increased glass stability at a low pH, a dilute hydrochloric acid wash was selected to wash glass-bead-immobilized catecholamines (373, 374, 379, 399). The importance of acid washing for the removal of noncovalently coupled catecholamine from catecholamine-Sepharose preparations was also documented (365). The release rates of catecholamines from preparations washed with acid until no parent amines were associated with the glass beads are only 0.008%/hr at 20°C and dropped to 10^{-5}/hr at 4°C (373, 374).

Some examples of drug release curves from glass and Sepharose drug preparations are illustrated in figure 3. Drug leakage rates are a function of the stability of the bonds linking the drug to the matrix and the stability of the matrix itself. Therefore, the amount of free drug attained in a solution from solid support immobilized compounds will be a function of the drug leakage rates, the amount of drug coupled per immobilized unit (i.e., per bead) and the number of units or matrix particles present in a given volume. Whether the released drug will reach a pharmacological concentration will depend primarily on the above factors, the volume of the solution, and the tissue activation threshold for that drug. These conditions relate primarily to immobilized drug preparations where the starting "parent" drug has been essentially eliminated from the matrix.

In washing glass-bead-immobilized isoproterenol, the release products can be readily monitored by thin-layer chromatography. Material released from unwashed or briefly washed isoproterenol glass beads can be demonstrated to consist primarily of the parent, noncovalently coupled isoproterenol (373). Minor peaks are also identifiable as 6-aminoisoproterenol and a side arm azo-isoproterenol product. The 6-aminoisoproterenol is produced by reductive cleavage of the azo bond linking the covalently coupled isoproterenol to the glass (373). The ratio of the released products can change as a function of the degree of acid washing that the beads receive, so that after acid washing, the presence of parent isoproterenol is no longer detectable and 6-aminoisoproterenol and the side arm azoisoproterenol become the released products (176, 399). When the parent compound is present in a noncovalently bound form, such as isoproterenol contaminating the isoproterenol-glass bead preparations, or native insulin contaminating insulin-Sepharose preparations, no valid conclusions can be drawn concerning the biological activity of the covalently coupled species.

The leakage rate of drugs from matrices, as stated above, can be determined by many factors. The matrix stability is one major determinant of drug release. Sepharose drug preparations appear to be less stable than glass-bead-immobilized drugs. Even with acid washed

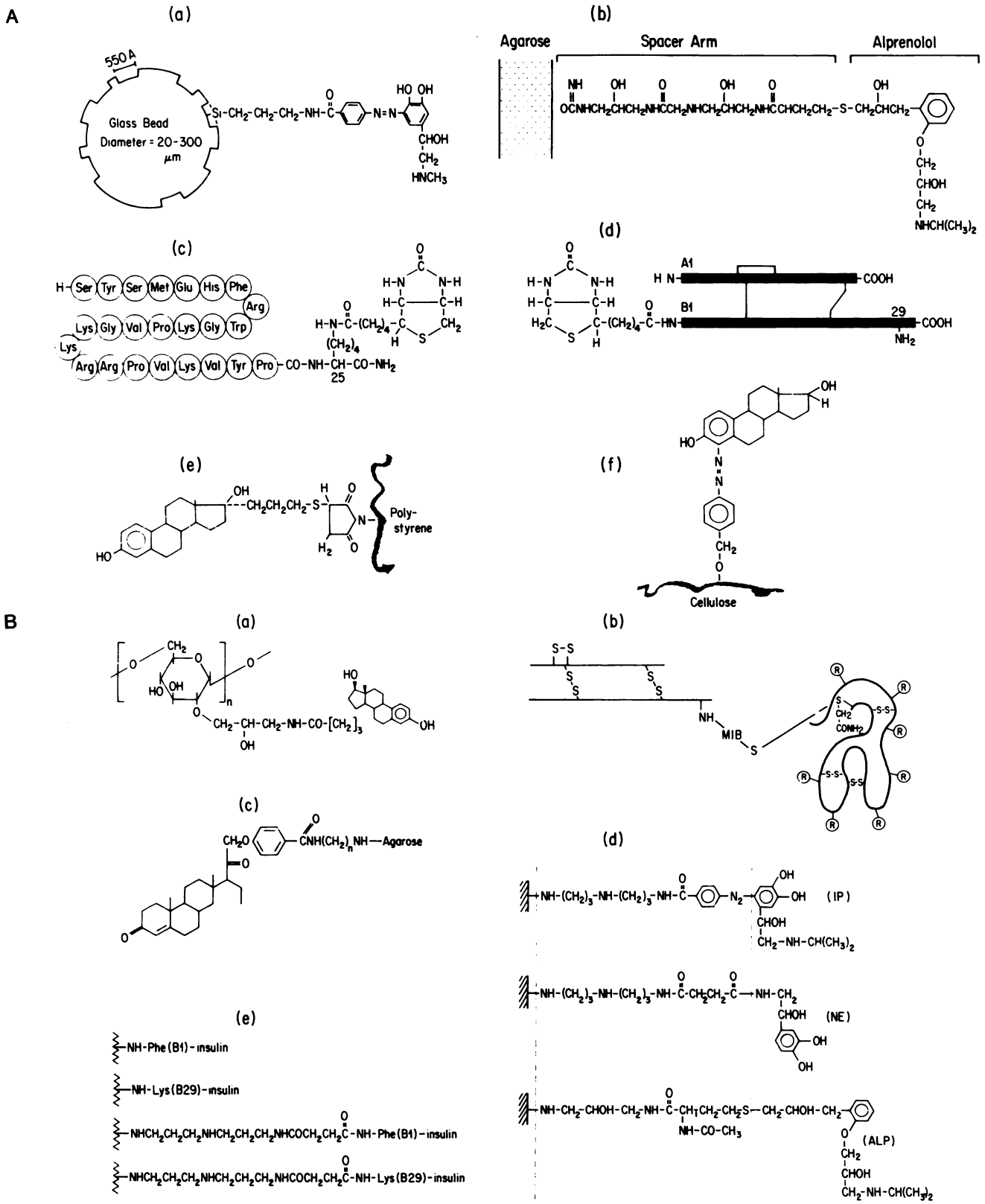

FIG. 2. Proposed structures of some immobilized drugs and hormones. A. (a) L-Epinephrine-glass beads redrawn from Venter et al. (375); (b) alprenolol-agarose redrawn from Vauquelin et al. (362); (c) [biotin^{25}] ACTH (1-25) amide redrawn from Hofmann and Kiso (144); (d) NαB^1—biotinylinsulin redrawn from Hofmann et al. (143); (e) polystyrene immobilized estradiol; and (f) *p*-aminobenzyl estradiol diazotized to *p*-aminobensyl cellulose, redrawn from Vonderhaar and Mueller (385).

B. (a) Dextran-estradiol conjugate redrawn from Herbert et al. (151); (b) rhodamine 7-α-lactalbumin-insulin conjugate; redrawn from Shechter et al. (322); (c) deoxycorticosterone immobilized on agarose; redrawn from Failla et al. (105); (d) isoproterenol (IP), norepinephrine (NE), and alprenolol (ALP) linked to agarose; redrawn from Vauquelin et al. (363); (e) insulin linked to agarose via the B_1 phenylalanine and B_{29} lysine to various length spacer groups; redrawn from Cuatrecasas and Parikh (77).

catecholamine-Sepharose preparations, the release rates at pH 7.4 are greater than 1% of the total bound amine per hour (365). The drug release rates from glass bead preparations appear to be lower. Various release rates of immobilized drugs from glass beads range from 0.006%/hr for growth hormone (29) to 0.008% (373), 0.11%, 0.2% (406), and 0.3%/hr (399) for catecholamine glass beads. All immobilized glass and Sepharose drug preparations have been reported to release soluble compounds. However, the experimental conditions used in testing the immobilized drugs are not necessarily restricted to mechanism A (fig. 1).

3. Experimental conditions for testing solid phase "immobilized drugs" for biological activity. In studies with glass bead catecholamines, mechanism A was eliminated from consideration simply by adding less total catecholamine in moles to the test systems than the minimum amount of drug necessary to achieve a threshold concentration in solution (176, 370, 375, 379–381). The threshold concentration for epinephrine to elicit a positive inotropic response in isometrically contracting cat papillary muscles is on the order of 10 nM or 0.5 nmol in a 50-ml bath. If the entire content of one epinephrine glass bead (6 pmol) (373) was released completely as epinephrine at one time into solution, a pharmacologically active concentration could not be attained. The drug leakage rate from one glass bead was determined to be on the order of 100 attomol/hr (373, 379). Therefore, the total amount of free drug available to the tissue is well below the limits necessary to eliminate mechanism A. A second approach utilized to rule out mechanism A demonstrates a requirement for physical contact between the catecholamine-glass beads and the effector tissue for biological activity. This was accomplished in a number of ways, for example, epinephrine-glass beads were attached to a glass rod, the rod in turn being connected to a micromanipulator, therefore permitting the epinephrine-glass beads to be placed at varying distances from the surface of the cardiac muscle. No muscle activation could be produced unless physical contact was established (375). Subsequent experiments established that cardiac muscle could propagate a response initiated by the surface localized catecholamine. Section V C 1 shows how the immobilized catecholamine led to the elucidation of this physiological mechanism.

In addition, direct reversal of aortic strip contraction, produced by up to 6,000 norepinephrine-glass beads, could be obtained by simply washing the beads off of the muscle, even if the catecholamine beads were allowed to remain in the muscle baths (21, 407). Several other studies have demonstrated that physical contact between catecholamine-glass beads and the muscle surface, and not merely the presence of catecholamine-glass beads in the muscle bath, is required for muscle activation (16, 21, 156, 375, 380, 381).

The same test conditions can be applied to Sepharose-drug preparations but the greater leakage rates restrict further the number of immobilized units that can be added to a test preparation.

With isolated whole and broken cell preparations, elimination of mechanism A becomes increasingly complex. Instead of having a single tissue mass in which responses may be propagated (371), there may be as many as 10^7 or greater single cells or tissue fragments (62, 375). If the stimulus in such a system is to be attributed to the immobilized form of the drug, then it would seem reasonable that a sufficient amount of the drug matrix would need to be added to the test systems to allow the immobilized drug to physically interact with the majority of cells present. It therefore seems unreasonable to assume that 10 or less glass or Sepharose beads could interact with adequate numbers of single cells under these conditions to elicit measurable responses (181). The binding of various tumor cells to drugs and hormones immobilized on glass and Sepharose beads has been studied (table 2). Utilizing radioactively labelled cells, one study calculated that an average of 10 to 20 cells can bind per Sepharose bead and up to 300 cells can bind to the average glass bead (369). Photomicrographs demonstrating glial tumor cell binding to azoisoproterenol glass and Sepharose beads are shown in figure 4. These data indicate that on the order of 10^6 Sepharose beads would need to be added to a test system of 10^7 cells for the immobilized drug to bind the majority of the cells. The instability of the Sepharose and glass preparations does not permit adequate assessment of immobilized drug action under these conditions. However, these or similar conditions have often been used to assess "immobilized drug" action (44, 62, 167, 190, 311, 365, 375, 395).

4. Mechanism of action of glass-bead-immobilized catecholamines. The problems associated with proving the mechanism of action of the glass bead-catecholamines are in many respects common to other solid phase immobilized-drug systems. As discussed in the following sections, soluble azo-substituted and polymer-immobilized catecholamines do retain substantial activity in the covalently coupled form. However, due to the physical nature and chemical stability of existing solid supports, the questions concerning biological action of covalently coupled species become infinitely more difficult to answer convincingly.

The elemental difference can be examined in terms of the comparison of 300 μm diameter glass bead to a 1,500 MW copolymer to which the catecholamine is covalently coupled; the glass beads, under standard synthesis conditions, will bind on the order of 10^{12} molecules of isoproterenol per single bead, whereas a single polymer chain binds less than one molecule of isoproterenol, on the average (149, 370). While the single bead is sufficient for isolated cardiac muscle activation when the bead is placed on the muscle surface, greater than 10^{16} molecules of polymer-isoproterenol are needed for muscle activation when the drug is given in solution. The release of all the isoproterenol molecules from the bead into solution

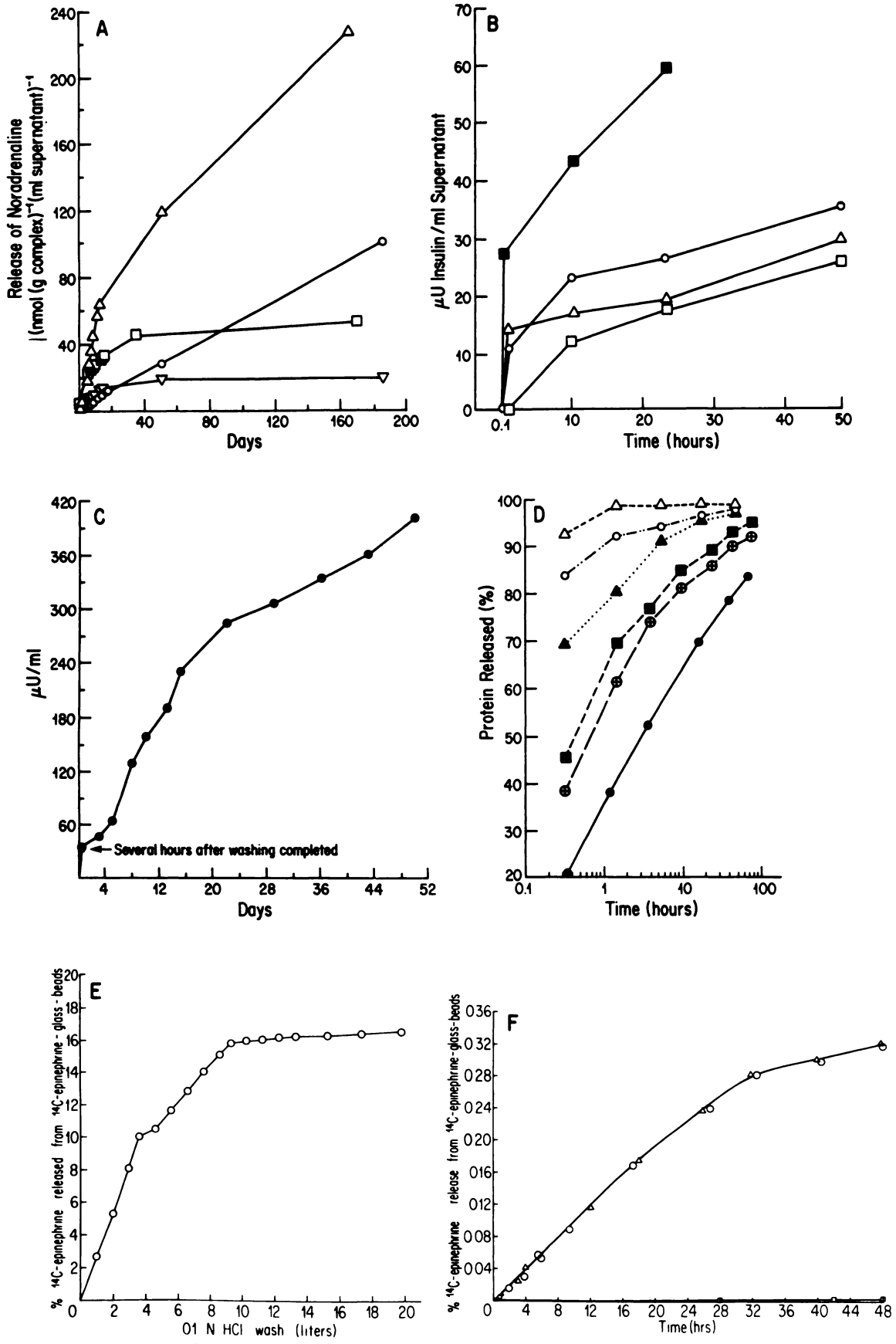

FIG. 3. Drug leakage rates from sepharose and glass-bead immobilized drugs. A. Release of [^{14}C]-norepinephrine from norepinephrine Sepharose, (△ and ○ and ▽); and from norepinephrine glass beads (□), over one half year; redrawn from Yong and Richardson (407). B. Liberation of soluble immunoreactive insulin from insulin-Sepharose in distilled H_2O at 4°C (□) in distilled H_2O at 20°C (■) in 0.02 M $NaHCO_3$ (pH 8.6) at

obviously cannot give the same effect as the soluble drug. The most stable isoproterenol-glass bead complexes release catecholamine at rates on the order of 0.008%/hr at 25° to 37°C (373) which for a single glass bead would represent approximately 23,000 molecules of catecholamine per second. It has been shown that the catecholamine release rates become slower with time but are essentially linear over short periods; therefore, if we assume the catecholamine is released at a continuous rate, the free drug diffusion away from the glass bead can be described within certain limits by the diffusion equation for a diffusing substance liberated at a continuous rate from a point source (fig. 5) (371). These considerations have been applied to the catecholamine release rates from glass beads (fig. 5). From this figure, it can be seen that the released catecholamine concentration falls rapidly within small distances from the bead. However, for distances less than 10 μm, the molar concentration asymptotically approaches the molar concentration of the source. Within 100 Å of the surface of the glass bead, the molar concentration of drug could be sufficient to explain the biological action of the glass beads. The glass bead side arms with the catecholamine attached vary from 16 Å to 32 Å in length which places the covalently attached catecholamines within the nonstirred zone containing released catecholamine derivatives (371). The existence of the various drug species within this zone makes distinction of the active species essentially impossible. Rapid mixing of the solution would not affect the free drug in the layer surrounding the beads as when a solid is placed into a well-stirred liquid, a stationary layer of liquid will envelop the solid (371). Values for the thickness of the stationary layer for isolated muscles have been estimated to be on the order of 100 μm with diffusion the determinant of events in this zone. While mechanism A (fig. 1) is easily eliminated for the glass bead catecholamines, the above data would restrict the mechanism to mechanism B (fig. 1).

With existing commonly used supporting matrices such as glass and Sepharose where even as little as 200 drug molecules can be released per bead per second at a continuous rate, it can always be argued on theoretical grounds that the leakage is sufficient to cause effector activation within 100 Å of the bead surface. The question of whether biologically active drugs exist in the covalently coupled form cannot therefore be proven directly. Direct proof of solid support immobilized drug action would be possible under conditions of a more stable matrix, with increased stability of the bonds linking the drug to the matrix and a decreased total amount of drug coupled per bead so that the overall drug release can be reduced two orders of magnitude.

5. Mechanism of action of Sepharose-insulin. The problems associated with Sepharose-immobilized drugs are even more substantial due to the decreased stability of the Sepharose matrix. The leakage rates in some cases approach or exceed 1%/hr (81, 365), making the dissociation of mechanisms A and B for Sepharose-immobilized drugs difficult to evaluate on this basis alone.

Following the study on the biological activity of ACTH-Sepharose by Sato and coworkers in 1968 (311), Cuatrecasas (62) reported in 1969 that insulin covalently coupled to Sepharose beads via the B_1-phenylalanine or the B_{29} lysine retained biological activity on isolated fat cells. Cuatrecasas proposed that because insulin-Sepharose could not penetrate the cells, the increased glucose utilization and suppression of hormone stimulated lypolysis in the isolated fat cells was a direct result of insulin interacting with cell surface receptors (62). Insulin-Sepharose preparations were reported to mimic the actions of native insulin on glucose transport and utilization. This claim was supported by other investigations. For example, Turkington in 1970 reported that insulin- and prolactin-Sepharose were able to stimulate RNA synthesis in mammary epithelial cells although no controls were reported that would have detected released hormone (361). In 1971, Blatt and Kim reported that insulin-Sepharose stimulated glycogen synthetase in minced tadpole liver tissues (24).

In the study by Blatt and Kim, a unique control experiment supported the claim of immobilized insulin action (24). Minced liver samples were placed in two incubation compartments separated by a 1.2-μm pore size Millipore filter. When native insulin was added to one compartment, the glycogen synthetase activity was stimulated in the connected chamber; however, when insulin-Sepharose was added, glycogen synthetase activity was increased only in the chamber containing the insulin-Sepharose (24). It was assumed that because insulin remained attached to Sepharose that it could not cross the Millipore filter.

Oka and Topper also demonstrated what appeared to be a unique action of insulin-Sepharose. Insulin-Sepharose increased the rate of α-aminoisobutyric acid accumulation in mammary epithelial cells from mature virgin mice, cells that did not respond to native insulin. Insulin

4°C (○) and in 0.02 M sodium citrate pH 5.0 at 4°C (△) with time; redrawn from Kolb et al. (190). C. Rate of insulin dissociation from insulin-sepharose beads over 52 days; redrawn from Davidson et al. (81). D. Release of soybean trypsin inhibitor from polyvinylalcohol as a sustained release preparation. The percentage of polymer in the casting solution was varied, △, 1.2%; ○ 4.8%; ▲ 6%; ■, 10%; ● 20%; ●, 10% sandwich. Protein concentrations in all casting solutions 12 mg/ml. Release characteristics were studied by incubating polymer pellets with lactated Ringers solution at 37°C. Released protein was assayed directly. E. HCl wash profile of [^{14}C]epinephrine-glass beads. One gram of [^{14}C]-epinephrine-glass beads was packed into small column and washed continuously for 6 days with 20 liters of 0.1 M HCl Aliquots of the wash were counted and the cumulative percentage release plotted. Redrawn from Venter et al (373). F. Effect of temperature on the release rate of (^{14}C)-epinephrine-glass beads. One gram of (^{14}C)-epinephrine-glass beads was incubated in 10 ml of distilled H_2O at 4°C (□), 23°C (○), or 37° (△), and the cumulative percentage release was determined with time; redrawn from Venter et al.(373).

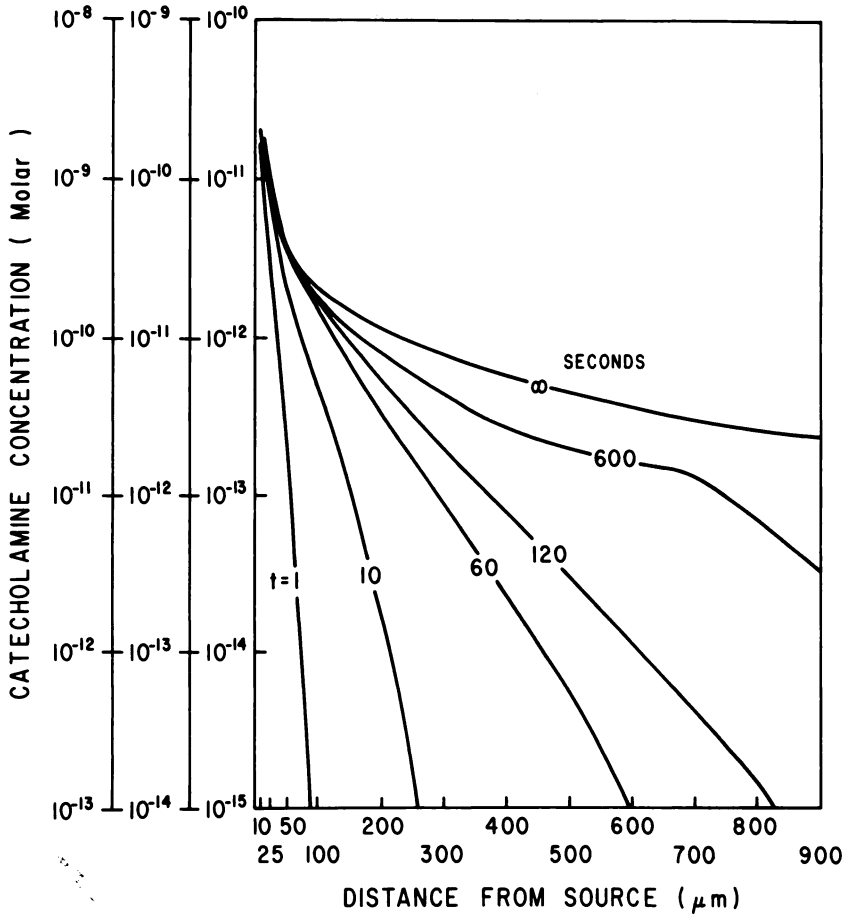

FIG. 5. Calculated diffusion of catecholamine into cardiac muscle from single catecholamine-glass beads. This curve represents calculated solutions for the diffusion equation

$$C = \frac{q}{4\pi Dr} \operatorname{erf} c \frac{r}{2(Dt)^{1/2}}$$

for a substance liberated at a continuous rate from a point source into an infinite volume. Values given on the ordinates represent (from left to right) concentrations from the solution of the equation for q=0.13, 0.013, and 0.0013 amole/sec. The curves indicate the molar concentration of drug obtained for different times from 1 sec to infinity. Values on the abscissae represent the distance into the muscle tissue from the single glass bead source of diffusing material. The values of q given above represent the maximum to the minimum experimentally determined catecholamine release rates from a single catecholamine-glass bead; reproduced from Venter (371).

instead blocked the action of insulin-Sepharose (248, 249).

In 1973 Davidson et al. (81) reported controlled studies on insulin-Sepharose action and concluded that the majority of previously published data on insulin-Sepharose action could be explained by the release of insulin from the Sepharose beads (81). Davidson et al. found in agreement with earlier studies that the final washes of insulin-Sepharose preparations contained no immunoassayable or biologically active material (24, 62, 81, 248, 249). However, when tyrosine transaminase levels were measured in cultured rat liver cells, the supernates from insulin-Sepharose preparations produced essentially as much activity as insulin-Sepharose itself (81). Immunoassayable material was found released under a variety of conditions (81). Insulin dissociation rates from Sepharose were determined over a number of days (fig. 3). Additional information was provided (81) on the binding of insulin to Millipore filters. This data has relevance to the studies by Blatt and Kim (24). Davidson et al. argued that the majority of insulin released from the insulin-Sepharose bead would be adsorbed by the Millipore

FIG. 4. Affinity isolation of cells with immobilized drugs. A. a and b, Phase contrast photomicrographs of the binding of C6-glioma cells to isoproterenol-glass beads (a) or to isoproterenol diazotized to hexylarylamine Sepharose (b) (369). c and d, Scanning electron micrographs of N1L2K cells, control (c) and associated with glycolipid-glass beads (d). The association of the glycolipid-glass couples with the cells induced glycosylation of the immobilized glycolipids in contact with the cell surface; reproduced from Yogeeswaran et al. (405). e and f, Namarski interference contrast microscopy of isolated rat epididymal fat cells associated with insulin-Sepharose beads. Largest sphere in each figure is the Sepharose bead, smaller spheres are the fat cells. Reproduced from Katzen and Soderman (180). g and h, Photomicrographs of mixed human leukocytes and erythrocytes in the presence of rabbit serum albumin-Sepharose (g) and histamine-rabbit serum albumin-Sepharose (h). There was a selective attachment of leukocytes to the histamine-Sepharose. Reproduced from Melmon et al. (224).

filters used to separate the two chambers and therefore never gain access to the second chamber (81). Furthermore, it was argued that the native insulin used as a control by Blatt and Kim was added to the first chamber at a high enough concentration (0.48 U/ml) to obtain at least 30 mU/ml in the second chamber and therefore would account for the enzyme stimulation in both chambers (81). These studies clearly demonstrated the importance of dose-response relationships in immobilized drug studies and how insulin-Sepharose data could be potentially explained by the insulin release. Davidson et al., however, stated that "we do not maintain all of the activity of Sepharose-insulin preparations is due to free insulin present," a point supported with data on rat diaphragm preparations (81). Insulin-Sepharose containing 210 μU/ml of insulin demonstrated biological activity on the rat diaphragm muscle (81). The supernates from these assays contain only 42 μU/ml of insulin and demonstrated no biological effects (81). The insulin-like activity was therefore attributed to the insulin-Sepharose as the concentration of released insulin was below the limits of sensitivity in the rat diaphragm assay (table 4). However, while this type of experiment helps rule out mechanism A (fig. 1) for insulin-Sepharose action on the diaphragm muscle, it does not distinguish between mechanisms B and C (81).

Cuatrecasas reported using low insulin-Sepharose concentrations in his study (62); however, Katzen and Valhakes (181) calculated from his data (62) that only a fraction of a Sepharose bead could have been added to provide the insulin-Sepharose concentrations reported (62). Butcher et al. (39) extended these arguments and further questioned the interpretation of the data by Cuatrecasas (62).

Kolb et al. reported on several biological assays with insulin-Sepharose and found sufficient insulin in the supernates from each experiment to account for the activity of the insulin-Sepharose (190). Although it was concluded that insulin was coming off the Sepharose, apparently no attempt was made to determine the structure of the released material (190).

Oka and Topper (250) found that material with insulin-like activity was released from insulin-Sepharose preparations in the presence of bovine serum albumin (BSA). The insulin-like material released from Sepharose in the presence of BSA increased α-aminobutyric acid accumulation in mammary explants to a greater extent than did native insulin and was therefore referred to as superactive insulin (250). Wilchek et al. (397) determined the structure of the superactive insulin to be a covalent complex between BSA and insulin (fig. 6). The BSA-insulin complex displayed super-insulin activity in mammary explants and mice diaphragms (397). Soluble insulin complexes were also obtained by treating insulin-Sepharose with bovine casein (397). Soluble active forms of prolactin and placental lactogen have also been prepared from the Sepharose hormone by treating with BSA (386). Wilchek et al. (397) discussed how the reaction of the N-substituted isomer conjugates of Sepharose with amines and BSA helps to clarify the discrepancies among different publications regarding the stability of the (Sepharose-hormone) conjugates. "Some studies showed that the product is unstable while others have claimed conjugate stability. In the studies where leakage was observed, the buffer usually contained amines or BSA while in studies in which leakage was not observed, other buffers were used" (397). Data presented by Wilchek et al. demonstrate that insulin-Sepharose works as a covalent complex, albeit not as an insulin-Sepharose complex but most likely as an insulin-BSA complex (397).

Studies by Soderman et al. (180, 336) on the affinity binding of fat cells to insulin-Sepharose (fig. 4) demonstrated that insulin-Sepharose can interact with insulin receptors; however, the biological effects attributed to insulin-Sepharose may best be explained by insulin released from the Sepharose matrix perhaps in the form of a polymeric insulin BSA complex (397).

With other immobilized drug systems, Smith and coworkers have demonstrated that the action of Sepharose-immobilized cardiac glycosides can best be explained in terms of glycoside leakage (334). A series of cardiac glycoside derivatives attached to glass and Sepharose beads via a variety of linkages were synthesized (370). After washing, the glass bead glycoside derivatives were inactive on isolated cat papillary muscles. In vitro inhibition of sodium-potassium ATPase could be explained completely by the amounts of free glycosides released from the glass beads.

Also consistent with the findings of Wilchek et al. (397) are those of Vauquelin et al. on the action of catecholamine-Sepharose preparations (365). When Sepharose-catecholamine beads were acid washed so that the con-

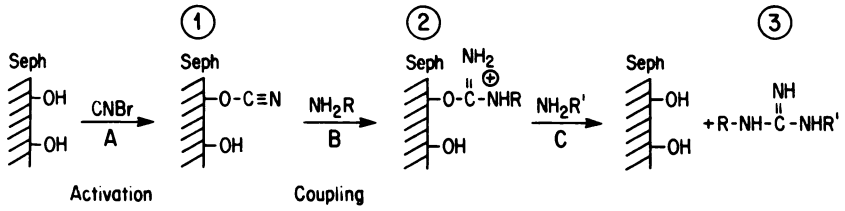

FIG. 6. Formation of "super-active" insulin. Activation of Sepharose (Seph), coupling with amines to form Sepharose-bound isoureas and the release of substituted guanidines. Superactive insulin is an N_1—N_2—disubstituted guanidine in which insulin = R and bovine serum albumin = R'. From Wilchek et al. (397).

tamination by parent amine was low, the principal release product was shown to be the Sepharose side arm with the azo-substituted catecholamine attached (365).

C. Direct Evidence for Covalently Immobilized Drug-Receptor Interactions

The direct binding between an immobilized drug and its receptor is generally a readily testable parameter. The majority of the evidence for immobilized drug-receptor interactions emanates from the affinity chromatography literature. Some of the receptors and cell systems that have been reported to bind directly to ligand-matrix systems are summarized in table 2.

Criteria for "biospecific" affinity chromatography have been discussed (63, 67, 73, 162, 246). The term "biospecific" affinity chromatography is used to describe a situation where the cell or macromolecule in question is adsorbed specifically by binding to an immobilized receptor ligand in a chemically specific manner is as expected from known ligand-receptor interactions. Biospecific adsorption is to be distinguished from "nonspecific" adsorption which depends more on the gross physiocochemical properties of the system. These properties include such parameters as the net charge of the macromolecule or cell and hydrophobicity of the system. Many of the existing reports on affinity chromatography are thought to be complicated by nonspecific interactions (246). O'Carra et al. (246) have demonstrated how biospecific retardation (R_{bio}) of an enzyme or receptor on an immobilized ligand can be related to the molar dissociation constant (K_{im}), and the effective molar concentration, (Im) of the immobilized ligand by equation I.

$$R_{bio} = (Im)/K_{im} \quad (1)$$

The addition of a competing ligand to the system will produce a decrease in the biospecific retardation depending upon the dissociation constants and concentrations of the immobilized and competing ligands (246). Systems should display temperature and pH dependence in accord with the temperature effect on association and dissociation rates of the ligand for the receptor or enzyme in question.

With membranes and intact cells, demonstration of biospecific adsorption is further complicated by the possibility of multiple receptor interactions with the immobilized ligands. These multiple site interactions explain in part why the use of competing ligands to desorb cells from affinity matrices has met with little success. However, prevention of cell adsorption by prior receptor occupation with competing ligands is an alternate method of demonstrating biospecific interactions.

In addition to affinity isolation of receptors and cells, the interaction of receptors with immobilized ligands can give important clues as to structure activity relationships involved in ligand-receptor interactions.

1. Nicotinic acetylcholine receptor interactions with immobilized cholinergic ligands. The use of Sepharose immobilized cholinergic ligands has had a major impact on the purification of nicotinic acetylcholine receptors from numerous sources. Conventional biochemical techniques that were initially applied to the purification of the acetylcholine receptor met with only limited success. This was attributed to low levels of purification and receptor desensitization (116).

The most common method for receptor purification has been affinity chromatography with a cholinergic ligand covalently coupled to a solid support (table 2). Acetylcholine receptor proteins have been purified from the electric organs of a variety of fish (23, 32, 47, 48, 100–102, 136, 140, 174, 177–179, 188, 212, 231, 253, 255, 264, 292–294, 296, 304, 314, 335, 344, 392), skeletal muscle (33, 87, 106, 115), and cultured muscle cells (30, 235). In several studies acetylcholine receptors have been selectively adsorbed to Sepharose columns containing α-bungarotoxin, quaternary ammonium ligands, or other cholinergic ligands (table 2). Acetylcholine receptors were recovered from the columns by "specific" displacement with cholinergic ligands such as decamethonium (23, 230), hexamethonium (174, 188), α-toxin (292), flaxedil (231, 253, 344), carbamylcholine (100, 101, 135, 177–179, 255, 325, 392), benzoquinonium (100, 264), and 3,3′-(bis(α-trimethylammonium)methyl-azobenzene (bis Q) (47). Although the attainment of highly purified receptors from these affinity columns may argue well for the specificity of the interaction, the concentrations of eluting ligands were often in the millimolar range for most antagonists (23, 100, 253, 264) and in the molar range for agonists such as carbamylcholine (33, 100, 101, 135, 179). An exception to these high concentrations for eluting ligands is from the study by Chang on the purification of the acetylcholine receptor from *Electrophorus electricus.* Chang utilized a phenyltrimethylammonium affinity column and eluted the receptor with 3 μM bis Q (47).

In addition to cholinergic ligands, Raftery and coworkers have utilized NaCl gradients up to 0.5 M to elute the receptor from quaternary ammonium affinity columns (294, 314, 315, 335). The fact that salt displaces the receptor in similar concentrations to some cholinergic agents suggests that the affinity step may not be all that specific. There are reported differences in the receptor elutions from the different columns. The sodium chloride elution, for example, works only with quaternary ammonium ligands bound to Sepharose and not with α-toxin -Sepharose columns (116, 314, 315).

Although the resultant purification of the acetylcholine receptor by affinity chromatography is highly indicative of receptor specific interactions with immobilized ligands, the receptor molecules in these studies were in a soluble state and therefore possibly more accessible to the immobilized ligand.

More convincing evidence for interaction of immobilized cholinergic ligands with membrane-bound acetylcholine receptors comes from the studies of Flanagan et al. (108, 109), in which receptor-containing membranes

were purified by affinity phase partitioning (107–109). Affinity phase partitioning is a modification of the phase partitioning method of Albertson (3) developed by Flanagan and colleagues (107–109). In phase partitioning a distribution or partitioning of substances into different aqueous phases, obtained by mixing aqueous solutions of two suitably different polymers, can be obtained as a function of polymer charge, ionic strength, pH, and hydrophobic character of the polymers (3, 35, 107–109, 133, 389). A typical system employs high molecular weight dextran and polyethylene glycol which separate into phases above a certain "critical" concentration. Macromolecules, membranes, or cells will partition into either the dextran rich or the polyethylene glycol rich phase as a function of the parameters discussed above. In affinity phase partitioning additional selectivity is obtained by the covalent attachment of an affinity ligand to one of the polymers introduced into the system (107–109). In the studies by Flanagan et al. (108, 109) trimethylammonium ligands covalently attached to polyethylene oxide polymers effected the partitioning of acetylcholine receptor rich membranes in the phase system by a specific interaction with the acetylcholine receptor (108, 109). The immobilized ligand-polymer dependent phase shifts were inhibited by bisquaternary methonium ligands at concentrations consistent with their relative affinities for the cholinergic receptor (fig. 7) (108, 109). Specific blockade of receptor sites with α-bungarotoxin decreased the distribution changes in a stoichiometric fashion (108, 109). Similarly charged polymers lacking specific receptor binding properties did not effect receptor partitioning (108, 109).

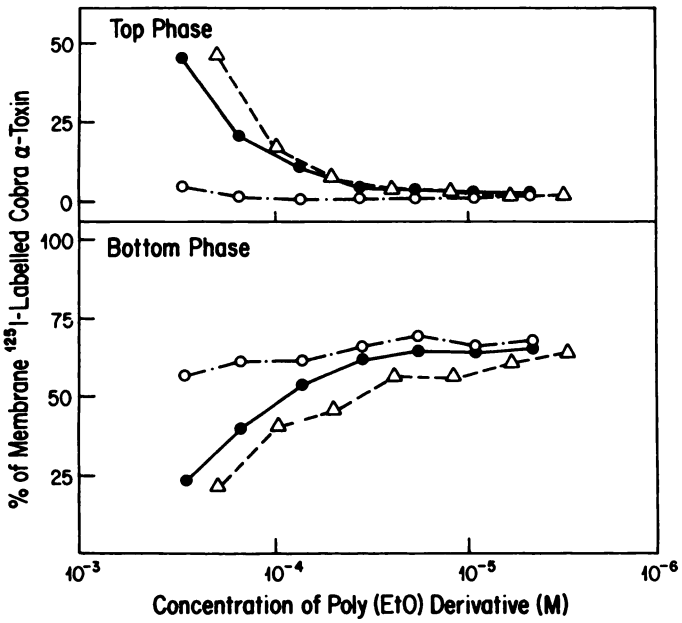

FIG. 7. Affinity phase partitioning of acetylcholine receptor enriched membranes. Phase partitioning diagrams illustrating the distribution of cholinergic receptor enriched membranes derived from electroplax of *Torpedo californica* in a two-phase polymer system containing polyethylene oxide (poly EO) and dextran. Affinity partitioning was accomplished by the addition of ligand substituted polyethylene oxide that "pulled" the receptor enriched membranes into the polyethylene oxide rich phase. The figure illustrates the distribution of membrane-bound ^{125}I-labelled α-bungarotoxin in the presence of various concentrations of α-ω-bis methylamino poly EO (○); α-ω-bistrimethylammonium poly EO (△). The trimethylammonium (cholinergic ligands) pulled the acetylcholine receptor containing membranes into the top phase in a concentration dependent manner. Redrawn from Flanagan et al. (108,109).

2. β-Adrenergic receptor interactions with immobilized adrenergic agents. Preliminary studies reporting on the successful application of affinity chromatography to the purification of β-adrenergic receptors have appeared (45, 112, 362, 376–378). Some of these studies utilized the β-adrenergic antagonist alprenolol covalently attached to Sepharose as the affinity adsorbant (45, 362). Due to instabilities of solubilized mammalian β-receptors (342), the affinity chromatography appears to be primarily of benefit with nonmammalian sources of receptor such as frog (45) and turkey erythrocytes (112, 362, 376). While there are similarities in the approaches taken by different laboratories that use alprenolol affinity chromatography (45, 362), the study by Caron and coworkers (45) provides evidence for "biospecific" affinity adsorption. Adrenergic agents were effective in inhibiting receptors from being adsorbed to alprenolol columns in accord with their affinity for the receptor with stereospecificity being retained (45). A concentration of only 1 μM alprenolol was required for maximal elution of receptors from the column and the β-receptor agonist isoproterenol was maximally effective at a concentration of 100 μM. Stereospecificity was also demonstrated for agonist and antagonists in receptor elution (45). This study contrasts to that of Vauquelin et al. where 1 M NaCl was required for receptor elution in addition to adrenergic ligands (362).

Mammalian β-receptors can bind specifically to both agonists and antagonists immobilized on Sepharose and the receptor adsorption can be inhibited by receptor occupation by adrenergic antagonists; however, receptor instability has prevented receptor recovery from the affinity columns (112, 342, 376; Segal and Venter, unpublished observation). Fraser and Venter (112, 376) have utilized monoclonal antibodies developed against β-receptors as immunoaffinity reagents. The monoclonal antibodies that were coupled to Sepharose 4B provided specific adsorption of partially purified turkey erythrocyte β-receptors. The antibody that is directed against a determinant in the adrenergic ligand binding site competed with adrenergic antagonists for binding. This unique arrangement provided the means of a biospecific elution of the receptors from the immunoaffinity column (112, 376, 377). β-Receptors were eluted with 1 μM 1-propranolol (112, 376, 377). Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of purified material indicated a molecular weight of 70,000 daltons for the turkey erythrocyte β-receptor (112) in contrast to lung β_2-receptors which have a molecular weight on the order of 59,000 daltons (376).

The studies on soluble β-receptor interactions with immobilized adrenergic agents and monoclonal antibodies were preceded by investigations on the interaction of intact cells (225–230, 369, 395) or membranes (378) with immobilized catecholamines. In a series of studies by Melmon, Bourne, and coworkers (225–230, 395), the interaction of lymphocytes with norepinephrine-Sepharose affinity columns was investigated. Lymphocyte adsorption to norepinephrine-Sepharose was inhibited by prior occupation of cell receptors with various adrenergic ligands. Adrenergic antagonists were successful in eluting the cells from the Sepharose beads (225–230, 395).

The specific adsorption of cultured tumor cells to isoproterenol covalently bound to Sepharose and glass beads was also reported (369). Prior β-receptor occupation by propranolol blocked cell adsorption (369). A correlation appeared over a series of cells in their ability to bind to the isoproterenol-beads and their responsiveness to catecholamines (369).

In 1974, studies with β-receptor containing turkey erythrocyte membranes demonstrated a 142-fold purification of isoproterenol stimulated adenylate cyclase specific activity by binding the membranes to isoproterenol glass beads (378). β-Receptor occupation by propranolol blocked membrane adsorption to the beads (378). During membrane binding to the isoproterenol-glass complex, adenylate cyclase appeared to be maximally activated (378).

Affinity phase partitioning has been applied to β-receptor rich membranes isolated from cultured human lung (VA_2) cells (Venter, unpublished data). In these studies isoproterenol was coupled to polyethylene glycol (PEG) via an azo linkage, and added to a two-phase polymer system (107–109). Membranes enriched in β-receptors, as determined by ^{125}I-iodohydroxybenzylpindolol(IHYP) specific binding, were obtained in the PEG-rich phase as a function of the amount of isoproterenol-PEG added. Receptor occupation by adrenergic ligands inhibited the phase shift. These studies with isolated membranes, where enhanced β-receptor binding was obtained from a purified membrane preparation, suggest that β-receptors may exist in clusters on some cell surfaces.

In other studies, polymeric immobilized isoproterenol was shown to compete for IHYP binding in isolated membranes (Venter, unpublished observation); and polymeric immobilized epinephrine was found to compete for specific α-adrenergic receptor binding on liver membranes (85).

Meier and Ruoho have made use of the avidin-biotin system (sections II and III) in β-receptor research by synthesizing a biotinyl-propranolol complex which interacts with duck erythrocyte β-receptors (223). Although the biotinyl-propranolol and the avidin-biotin-propranolol complexes had reduced affinity for the erythrocyte receptor, these complexes may have promise for labeling cell surface receptors.

3. Immobilized insulin and insulin receptors. Macromolecules with insulin binding properties have been solubilized from a number of mammalian tissues (65, 66, 68, 74, 77, 137, 159, 160, 198, 200, 219, 268). These macromolecules, which have been demonstrated to be insulin receptors (e.g., see Kahn 170, 171 for a review), bind to insulin covalently attached to Sepharose beads (65, 66, 74, 77, 137, 158–160, 200, 219, 237). In a series of studies, Cuatrecasas and coworkers (65, 66, 74, 77, 158–160) utilized insulin Sepharose affinity columns to partially purify the insulin receptor. Occupation of receptors by insulin prior to exposure to insulin-Sepharose prevented the receptor from being retained by the columns (66). Jacobs et al. reported that an insulin-Sepharose chromatography step provided a 2000-fold increase in the specific activity of an insulin-binding macromolecule from rat liver (160).

Studies by Czech and coworkers (137, 268), Harrison and coworkers (200), and Cuatrecasas and coworkers (159) have yielded highly purified insulin receptors for subunit structure identification.

In studies with intact fat cells and membrane ghosts, Katzen and coworkers (180, 336) have demonstrated that insulin-Sepharose can interact directly with cell surfaces. Katzen and coworkers capitalized on the unique buoyant density of fat cells which, when bound to Sepharose insulin, "floated" the Sepharose beads (fig. 4). The interaction of fat cells with insulin-Sepharose was inhibited or blocked by prior incubation of the insulin-Sepharose beads with anti-insulin antibodies or by incubation of the fat cells with insulin (180, 336). Glucagon, prolactin, BSA, leutinizing hormone, and gelatin at concentrations up to 40 ng/ml had no effects on the insulin-Sepharose fat cell interaction, whereas various growth hormone preparations at 4.8 ng/ml were partially effective (180, 336). Trypsin treatment of fat cells or insulin-Sepharose abolished the interaction (180, 336). These data suggest a specific interaction with insulin-Sepharose complexes and a cell surface component.

In more recent studies, Schechter et al. demonstrated specific interactions of covalent complexes of rhodamine-α-lactalbumin-insulin. These complexes were purified by gel filtration and tested directly for their affinity for adipocyte insulin receptors in competitive binding studies against iodinated insulin (322). Rhodamine insulin had 89% of the binding affinity of insulin and 75% of the biological potency (322). These direct binding studies demonstrate that covalently substituted insulin can interact with cell surface insulin receptors and that the binding is related to biological activity although perhaps not directly.

A different method for labelling insulin receptors with covalently substituted insulin complexes has come from the approach Hofmann and coworkers first applied to ACTH (144). The B-complex vitamin biotin was covalently coupled to the carboxyl terminal of ACTH and the complex purified. The covalent complex retained essen-

tially full ACTH-like activity in stimulating steroidogenesis in adrenocortical cells (144). Hofmann and Kiso (144) demonstrated that biotin-ACTH was a bifunctional molecule with affinity for both the ACTH receptor and for avidin. Avidin, a constituent of egg white, has an extremely high affinity ($K_d 10^{-15}$ M) for biotin (126). Hofmann et al. (143) and May et al. (220) reported on the successful synthesis of a stable covalent biotin-insulin complex. Hofmann et al. (143) attached the biotinylinsulin to avidin-Sepharose covalent complexes utilizing the affinity of biotin for avidin and demonstrated biological activity of the Sepharose-avidin-biotinylinsulin complexes. Ferritin-avidin complexes were proposed to specifically "stain" biotinylinsulin-receptor complexes on the surface of cells (220). A stoichiometric complex of insulin, biotin, and avidin retained the ability to produce maximal insulin responses in isolated fat cells (220). These approaches are clearly promising and warrant further investigation.

4. Estrogen receptors: affinity chromatography and affinity phase partitioning. Estrogen receptors, which are soluble macromolecules found in the cytoplasm and nucleus of target cells (401), have been partially purified by a variety of receptor affinity techniques (150, 151, 235, 328, 329, 385). Sica and coworkers (256, 329) successfully applied estradiol covalently attached to Sepharose to the partial purification of estrogen receptors. Estradiol covalently bound through the A-ring of the steroid was unable to specifically adsorb estrogen receptors (328) whereas steroid derivatives, e.g., 17 β-estradiol 17-hemisuccinate, bound to the solid support via the 17-position of the estradiol molecule were effective receptor adsorbents (328, 329). Direct receptor competition studies with ^{3}H-estradiol demonstrated that the hormone derivatives attached to Sepharose via the 17-position retained substantial affinity for the receptor (328). Receptors were desorbed from the columns with 10 μM estradiol and increasing the temperature from 4°C to 30°C (328, 329). One molar K^+ was ineffective at removing the receptors (328, 329).

In 1976 Hubert et al. (150) reported on the successful application of affinity phase partitioning to the purification of $\Delta_5 \rightarrow {}_4$ 3-oxysteroid isomerase (EC5.3.3.1). Estradiol was covalently coupled to polyethylene oxide via the 7-position (150). The K_I of the estradiol-polyethylene oxide complex was determined directly to be 5 μM (150). The presence of the hormone polymer complex in a polymer two-phase system substantially altered the partition coefficient for the isomerase while having little effect on total protein, therefore resulting in a partial purification of the enzyme (150). In 1978 Hubert and coworkers (151) applied a similar approach to the estrogen receptor. In this study estrogen was covalently attached to 500,000 MW dextran via the 7-position of estradiol (151). The covalent estradiol-dextran complex was shown in direct receptor binding studies to have a K_d of 0.3 μM at 0°C for the estrogen receptor compared to 0.2 nM for estrogen (151). The binding of estradiol-dextran to the estrogen receptor was readily reversible by competition with free estradiol (151). Estrogen receptors were partially purified by forming a receptor-estradiol-dextran complex by incubation of estradiol-dextran with receptor-containing fractions, followed by gel filtration over an Ultrogel ACA 34 column (151). This procedure resulted in the separation of the receptor-estradiol-dextran complex from the bulk of protein in the crude receptor preparation. Partially purified receptor was then recovered from the polymer complex by competing with ^{3}H-estradiol followed by a second gel filtration step (151).

Heparin-Sepharose has also been found to have a specific affinity for one form of the estrogen receptor (235) and the use of a heparin-Sepharose affinity column provided greater than 100-fold purification of the receptor (235).

In a related study, Kallos et al. (175) reported that estradiol-estrogen-receptor complexes associate in a competitive, reversible manner with DNA-cellulose. In direct competitive binding studies it was found that the drug-receptor complex had enhanced affinity for bromodeoxyuridine substituted DNA (175). The increased affinity of the estrogen receptor for the substituted DNA was interpreted as further evidence for a role for bromodeoxyuridine in modulating gene expression by binding to a regulatory protein (the estrogen receptor) (175). Estrogen receptor-DNA interactions and possible estrogen receptor-"acceptor" protein interactions have been reviewed [e.g. Yamamoto and Alberts (401)].

5. Direct interactions of other immobilized hormones with their receptors. Epidermal growth factor (EGF), like insulin, has been successfully conjugated with rhodamine-α-lactalbumin by substitution on the α-amino group of EGF (342). The rhodamine-α-lactalbumin-EGF complex retained 100% direct binding affinity for EGF receptors on cells and approximately 40% of the biological activity of EGF (342). The covalent fluorescent complexes containing EGF were utilized to localize and determine the fate of cell surface EGF receptors in 3T3 cells (312, 342).

Morphine and morphine analogs have been covalently coupled to glass (331) and Sepharose beads (330–332). The opiate-solid supports, while not capable of producing morphine-like responses on isolated guinea pig ileum (332), were capable of adsorbing antimorphine antibodies (332) and opiate receptor-containing membranes (330).

In a series of studies, the interaction of immobilized histamine with various cells (34, 224, 229, 277, 298, 320, 395) was investigated. In the initial studies by this group, histamine was covalently coupled to rabbit serum albumin which was in turn attached to Sepharose beads (224, 395). These insoluble histamine-containing complexes bound human leukocytes but not red blood cells or platelets (224, 395), whereas rabbit albumin-Sepharose without histamine bound no cells (224, 395). Histamine receptor antagonists prevented the leukocytes from bind-

ing to the histamine-RSA-Sepharose beads but had no effect on the cells binding to norepinephrine-RSA-Sepharose conjugates (224, 395). Cells that passed through histamine-RSA-Sepharose columns had reduced responsiveness to histamine (227, 229).

Growth hormone-Sepharose columns have been reported to have utility in the partial purification of prolactin receptors from rabbit mammary tissue (326). The affinity step produced up to a 200-fold purification but required 5 M $MgCl_2$ for receptor elution. Albumin-Sepharose and growth hormone-Sepharose, where the growth hormone was coupled directly with cyanogen bromide to Sepharose, were ineffective in retaining the soluble prolactin receptors (326). Prolactin receptors were retained by the affinity columns only when growth hormone was coupled to a side arm attached to the Sepharose backbone (326).

Affinity chromatography has also been applied successfully to gonadotropin receptors with either luetinizing hormone or chorionic gonadatropin covalently coupled to Sepharose (e.g. 95 and 97). Affinity procedures have also been applied to intact cells with ACTH and thyroid hormone covalently attached to glass and Sepharose beads (369).

Because of the successful application of immobilized drugs and hormones to the isolation of specific cells and receptors it is widely accepted that drugs can retain receptor specific activity in the covalently immobilized form. While this is clearly the case for most receptor isolation studies and for the biological activity of many polymeric drugs (section III), direct proof is lacking for biological activation studies utilizing solid phase immobilized drugs.

TABLE 5
Some pharmacological uses of polymeric drugs

Proposed Use	Reference
Chemotherapeutic agents	172, 254, 288, 20, 28, 52, 84, 128, 129, 272, 309
Antimicrobial agents	129, 305, 309, 310
Antifungal agents	284, 309
Hypolipemic agents	290, 291, 394
Artificial sweeteners	58–60, 205, 348, 394
Nonabsorbable food colors	17, 118, 148, 205, 206, 394
Interferon inducers	210, 279, 280, 272
Antithrombogenic agents	285
Immune system modulators	289, 272, 367
Antiviral agents	281–283, 10, 128, 243, 269, 272, 270, 275, 367
Plasma expanders or blood substitutes	350, 338
Cardiac stimulants	123, 176, 383
Antidiabetic agents	12, 22, 55, 192, 128
Herbicides	5, 257
Antiparasitic agents	128, 272, 309
Antioxidants	207
Antihypertensive agents	38
Detoxification agents	41, 42, 92, 128
Fertility control	128
Insecticides	5, 257, 201
Antihyperlipoproteinemia agent	290, 291

III. Polymeric Immobilized Drugs

A. *Introduction*

Polymeric drugs and hormones covalently coupled to natural and synthetic polymers have numerous advantages over solid phase immobilized drugs and are receiving widespread attention from a number of areas. Polymeric drugs are being utilized as a new approach to food additives (17, 58–60, 118, 148, 205, 206, 348, 394), chemotherapeutic agents (20, 28, 52, 84, 128, 129, 172, 254, 288, 309, 356), tools for basis receptor research (149, 371, 383), and such diverse areas as insect management (257) and growth induction of fir trees (5). The term "polymeric drug" appears to encompass a broad area including biologically active polymers, depot or substained release preparations, and drugs that act while covalently attached to a polymeric backbone (84, 89–91, 128, 129, 161, 172, 173, 176, 192, 202, 257, 297, 309). It is only this last class of polymeric drugs, those acting as carriers for covalently bound drugs, that will be discussed here. A partial list of polymeric immobilized drugs is given in table 3.

Wide ranging pharmacological properties have been attributed to polymeric immobilized drugs, some examples of which are listed in table 5; however, as with the solid phase immobilized drugs, one must be extremely careful in interpreting the experimental results. A conclusive demonstration that biological activity is associated with the polymer drug itself and not with contaminating free drug due to the breakdown of the polymer drug complex is required.

While such conclusive proof has been difficult to obtain with solid support preparations, the polymeric drugs provide unique circumstances which can circumvent many of the problems described in previous sections.

One of the principles for obtaining bioactive polymeric drugs pertains to having a low ratio or a stoichiometric relationship between drug molecules and polymer backbone (table 4). With only one drug substitution per polymer molecule, arguments pertaining to surface drug trapping and microenvironments of high concentrations of released drug (mechanism B, fig. 1), can be eliminated.

Due to a size difference between drugs and polymeric carriers, purification of the polymeric immobilized drugs from unattached drug molecules can be a relatively simple procedure with gel permeation chromatography. In studies with polymeric immobilized catecholamines, column chromatography provided not only a qualitative purification procedure but also a quantitative analytic step which allowed the determination of any catecholamine released under the various conditions of storage and biological testing (85, 149, 371 383, 384).

Enzymes have been immobilized on water-insoluble and soluble synthetic polymers such as dextran and copolymers of leucine with *p*-aminophenylalanine with

the clear retention of enzymatic activity (124). Immobilized enzyme cofactors such as the adenine nucleotides coupled to soluble dextran polymers have been shown to retain cofactor activity (131, 240).

In 1961 Arakawa et al. reported on the covalent attachment of angiotensin II and angiotensin analogs to polymers of O-acetylserine. The purified polymer angiotensin retained 41% of the pressor activity of soluble angiotensin. Based upon the polymer size of 28,000 daltons, it was concluded that angiotensin must work on cell membrane receptors (11). In 1971 Richardson and Beaulnes reported that angiotensin retained its activity while coupled to horseradish peroxidase and to cytochrome *c* without angiotensin dissociation (296).

Richardson and Beaulnes demonstrated immobilized angiotensin action based on a differential biological effect of the soluble and polymer immobilized angiotensin (296). Aortic strip contraction was prolonged when the immobilized but not free angiotensin stimulated muscles were immersed in oil (296).

In 1973 Regoli et al. (295) reported that angiotensin bound to poly O-L-alanine also retained activity. Cardiac glycosides coupled to albumin were reported to activate cultured heart cells (251) and insulin-dextran complexes demonstrated insulin-like activity (12, 169, 347, 351).

Drug release from the polymers and proteins can be argued to be the mechanism of action in many of the polymeric drug reports due to insufficient quantitation of free drug. However, for example, in studies by Suzuki et al. (347) on insulin-dextran preparations, sufficient quantitative studies with column purification of complexes were performed which illustrated that the biological action of insulin-dextran complexes was not complicated by the presence of released insulin.

B. Polymeric Immobilized Catecholamines

Studies with polymeric immobilized catecholamines (85, 149, 371, 383, 384) demonstrate a number of quantitative approaches to polymeric drug characterization. In these studies, *l*-isoproterenol, a *β*-adrenergic selective agonist (149, 371, 383, 384), or epinephrine (85) were covalently coupled via an azo linkage to various molecular weight random copolymers of hydroxypropylglutamine with *p*-aminophenylalanine (fig. 8). Amino acid analysis indicated that the final ratio of hydroxypropylglutamine to *p*-aminophenylalanine was 4.5 to 1 (149). The *p*-amino group was utilized in the diazotozation reaction and the phenylalanine residues which did not diazotize to the 6-position on the catecholamine ring of isoproterenol (373) were most likely reduced to tyrosine residues. Copolymer-catecholamine complexes had molecular weights of 1,500 to 13,000 daltons with one or less isoproterenol or epinephrine molecules per copolymer backbone (85, 149, 371, 383, 384). Copolymer-isoproterenol (copoly-Iso) was purified by gel exclusion chromatography over a Biogel P-2 column (fig. 9). With azo-linked catecholamines, azo-bond reduction produces a 6-amino

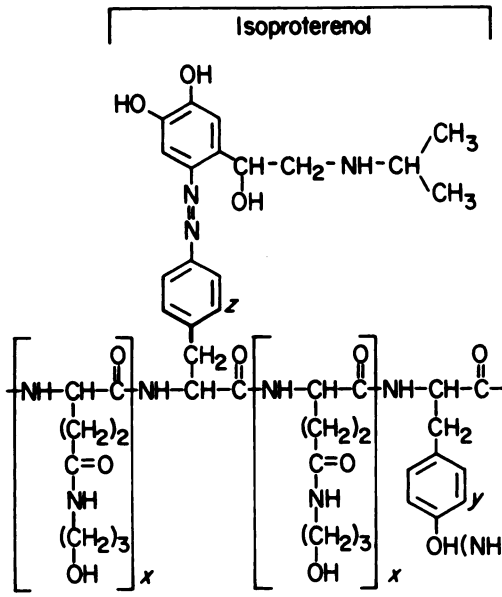

FIG. 8. Proposed structure of isoproterenol following diazotization to *p*-aminophenylalanine (z) in a random copolypeptide of hydroxypropylglutamine (x) with *p*-aminophenylalanine (y) (x:y = 4.5:1). The molecular weight is 12,800, estimated from ultracentrifugation in dimethylformamide (sedimentation and diffusion). There is 2.7% (by weight) incorporation of isoproterenol into the polymer. Since the diazotized aminophenylalanine residues are not fully substituted, the remainder are hydrolyzed to tyrosyl residues. From Hu and Venter (149).

derivative of the catecholamine (373); therefore, 6-aminoisoproterenol and parent isoproterenol would be the catecholamines expected to contaminate polymeric isoproterenol preparations. Due to minor ion exchange properties, the Biogel P-2 column clearly separated 6-aminoisoproterenol from the parent compound and the copolymer (fig. 9). The polymeric isoproterenol was rechromatographed until the parent catecholamine was below detectable limits (149, 383, 384). The highly purified polymeric-isoproterenol was tested for biological activity on isolated perfused guinea pig hearts (383, 384) and on isolated cat papillary muscles (149). The polymeric isoproterenol was nearly as active as the parent isoproterenol and substantially more active than 6-aminoisoproterenol (149, 189, 190). Rechromatography of material recovered from biological experiments indicated that only low percentages of 6-aminoisoproterenol were formed from the polymer and that no free isoproterenol was present (149, 189, 190). Responses of the magnitude found would require at least 5% contamination of the polymer with parent isoproterenol and 500% contamination with 6-aminoisoproterenol. These data together with diffusion and biological differences (149, 371, 383) indicate that catecholamines such as isoproterenol can provide biological activation of receptors while covalently attached to a supporting matrix.

Similar evidence has more recently been obtained by Dehaye et al. with polymeric immobilized epinephrine and liver α_1-adrenergic receptors (85).

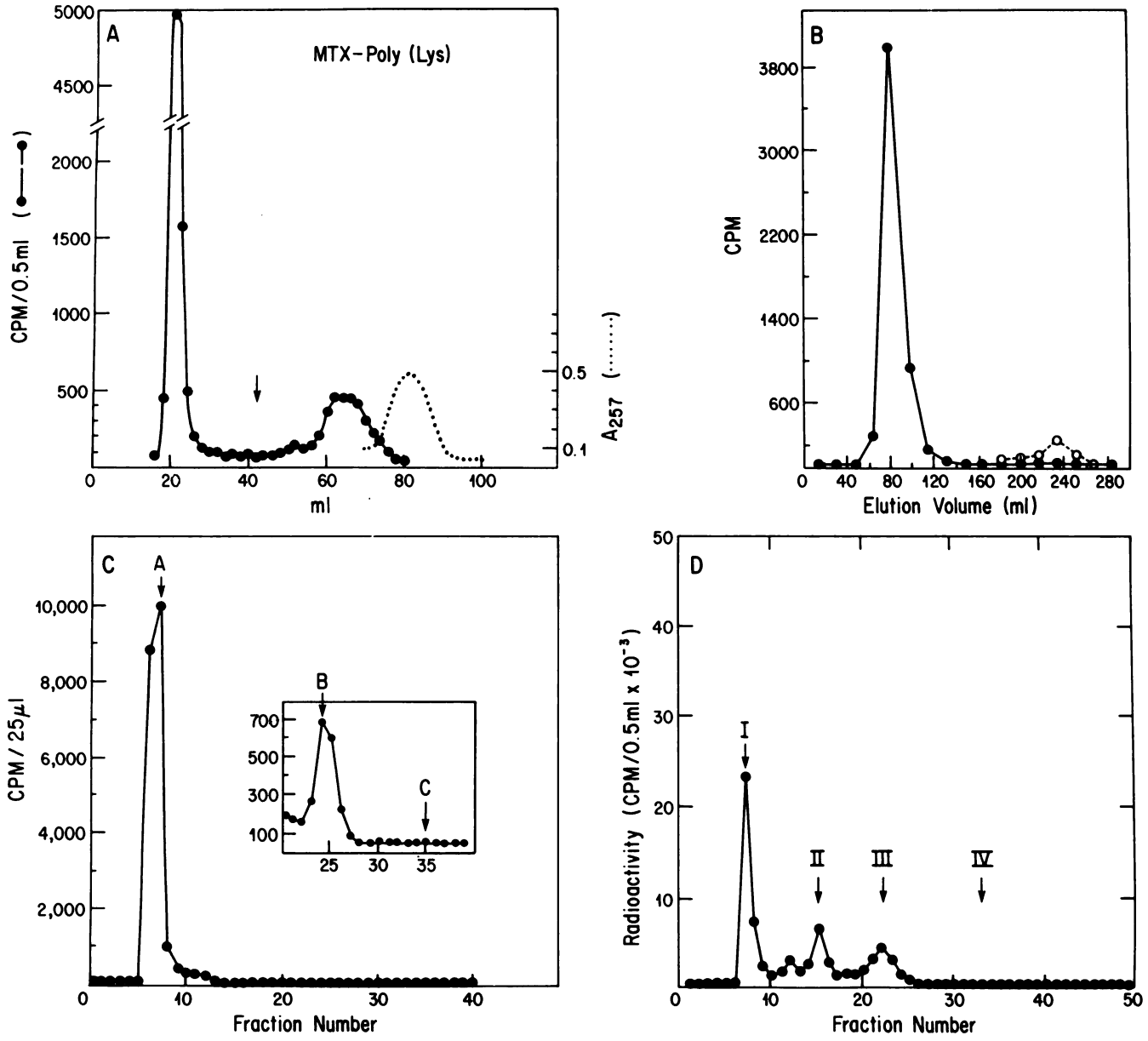

FIG. 9. Gel-permeation chromatography purification and analysis of polymeric immobilized drugs. A. Sephadex G-25 chromatography of cell lysates following a 24-hr incubation of Methotrexate (MTX) immobilized on poly L-lysine (MW 70,000). This experiment illustrated the intracellular breakdown of the polymeric methotrexate with the formation of a methotrexate degradation product (eluting between 60 to 80 ml) and close to free methotrexate (. . .). Redrawn from Shen and Byser (324) B. Separation of ouabain—BSA from free ouabain on Sephadex G-75. Closed circles illustrate the chromotography of ouabain—BSA which had been chromatographed twice previously with identical results; 50 ml portions of each sample were counted. Open circles show results of counting 1-ml samples with the clear presence of free ouabain. Redrawn from Smith et al. (334). C. Gel permeation chromatography purification of isoproterenol diazotized to a random 12,800 MW copolymer of hydroxypropylglutamine with *p*-aminophenylalanine (copoly-Iso). The elution profile for the separation of the copoly-Iso is shown in the main figure. The inset (40-fold increase in sensitivity) indicates a peak of 6-aminoisoproterenol. The peaks identified by thin-layer chromatography are copoly-Iso (A), 6-aminoisoproterenol (B), and 1-isoproterenol standard (C). Copoly-Iso was eluted from a column (Bio-Gel P-2, 3 × 120 cm) at 4°C with 1 mM ammonium bicarbonate. Fractions (13.9 ml) were collected and monitored by radioactivity. From Hu and Venter (149). D. Gel chromatography of polymeric azo-epinephrine. Elution profile following limited reduction of the azo-bond in polymeric azo-epinephrine (0.5 ml of a 0.1 μmM solution plus 2 mg of sodium dithionite and incubation at 50°C for 5 min). Peak assignment by comparison with known standards (I) polymeric azo-epinephrine, (II) 6-amino epinephrine, (III) adrenochrome like derivative of (II), and epinephrine (IV). These results demonstrated the stability of the polymer derivative and the complete absence of the parent epinephrine. Redrawn from De Haye et al. (85).

C. Targeting of Polymeric Drugs as Chemotherapeutic Agents

Models for the design of polymeric drugs have been proposed such as that by Ringsdorf (297) shown in figure 10. One of the principle concepts and theoretical advantages of polymeric drugs rests in the idea that a portion of the complex can be utilized to target a second bioactive portion to specific cells or tissues. This approach allows the design of tissue specific drugs to occur on a rational

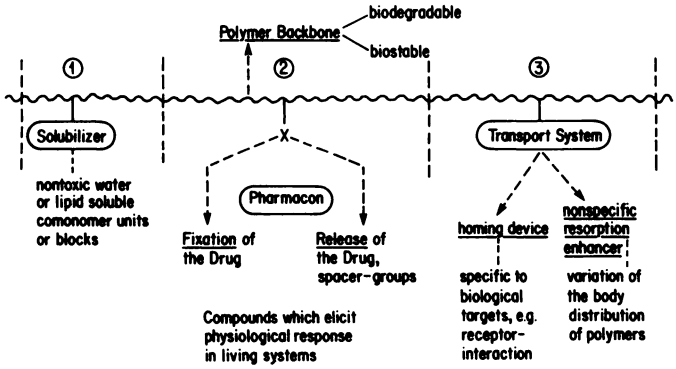

FIG. 10. Model for the design of polymeric drugs. Redrawn from Ringsdorf (297).

basis. While no polymeric drugs have yet proven themselves by clinical use on a wide scale, one area where these principles are being applied, in some cases quite successfully, is in cancer chemotherapy. A variety of antitumor drugs have been attached to a wide spectrum of site directing carriers. Chemotherapeutic agents have been attached to estrogen derivatives as a means of targeting breast cancer cells (368, 388, 103, 104). Some of these derivatives have shown a direct affinity for estrogen receptors (368), the ability to kill human breast tumor cells in animals (103) and in culture (368), and have been tested in clinical trials (104).

Many investigators have chosen antibodies as the site-directing species for cytotoxic agents. One of the first such agents resulted from the coupling of diptheria toxin to antibodies directed against cell surface antigens (238, 239). Philpott et al. in 1973 reported similar results (266). Increased toxicity and/or selectivity of polymeric cytotoxic drugs has been achieved in a number of recent studies (28, 46, 121, 122, 216, 234, 301, 354, 366, 402, 403). One of the more recent by Blythman et al. (28) utilizes only the A-chain of diptheria toxin and ricin coupled to anti-Thy 1,2 differentiation antigen monoclonal antibodies of the IgM class. The toxin A-chain-monoclonal-IgM complexes were toxic both in vitro and in vivo for antigen specific cells with "very low" toxicity for antigen negative cells (28). The A-chain of diptheria toxin has been coupled to lectins (121, 366, 400), to hormones such as insulin (234), epidermal growth factor (46), and the β-subunit of human chorionic gonadotropin (247), as well as to monophosphopentamannose (402).

Some of the more widely investigated polymeric drugs involve the chemotherapeutic agents daunorubicin, doxorubicin, adriamycin, and various nitrogen mustards which have been complexed with antibodies (119, 120, 153, 211, 305), dextran (20), synthetic polymers (303, 111, 155), and DNA (57, 359, 86). With the latter group, those where drugs are complexed with DNA, it is assumed that the targeting or cellular selectivity is due to the ability of some tumor cells to actively endocytose macromolecules, such as DNA. Daunomycin-DNA has reduced general toxicity with lethal effects on animal tumors (359, 360) and produced remissions of some malignancies in humans (57). In a series of studies it was shown that the chemotherapeutic activity of daunorubicin and doxorubicin are clearly increased when the agents are first complexed with DNA (83, 86, 359, 360).

The drug-DNA complexes fall into a category of agents defined as "lysosomotropic agents," agents which are selectively taken up into lysosomes (84, 161). Included as lysosomotropic agents are liposomes as drug carriers (127–129, 221, 259–261, 286). Liposomes have served as carriers for a large variety of drugs (127–129, 221, 286) and may be targeted by the attachment of drugs, hormones, or antibodies covalently bound to the outer surface (259). Additional types of lysosomotropic drug carriers are the asialoglycoproteins which appear to have high affinity for hepatic parenchymal cells (127, 129). Albumin and lysozyme coupled to asialofetuin were directed to the liver (299).

Methotrexate has been coupled to a variety of carriers including antibodies (52–54) and cellular proteins (54) and to various synthetic polymers (117, 289, 288, 306, 324) (table 3). Ryser and Shen (306, 324) reported that methotrexate covalently bound to poly (L-lysine) of various molecular weights (3,100 to 130,000 K) was able to overcome methotrexate resistance in tissue culture cells when the resistance was due to diminished methotrexate transport (306, 324). The conjugation with the polymer markedly increased the cellular uptake of methotrexate. The studies indicate that free methotrexate was released once the polymer was inside the cells as the polymer bound agent did not inhibit dihydrofolate reductase. The nondegradable poly (D-lysine) methotrexate complex did not release methotrexate intracellularly or inhibit cell growth (306, 324). The mechanism of action of the methotrexate polymer was demonstrated by examining the cellular lysates from treated cells with gel permeation chromatography as illustrated in figure 9. These results illustrate the need of a covalent complex between methotrexate and the polymer for increased cellular transport, with the final effect on the cell due to enzymatic release of the agent.

D. Other Therapeutic Uses of Polymeric Immobilized Drugs

As outlined in table 5 numerous therapeutic possibilities for polymeric drugs have been or are being investigated. The chemotherapeutic uses rely to a great extent on the targeting potential outlined in the previous section. However, other aspects of polymer carriers for drugs are under study. One area with exciting potential for polymeric drugs is in the field of food additives where the containment of agents such as artificial sweeteners and food preservatives to the gastrointestinal tract are desired goals. A review of this area by Weinshenker (394) discusses some of the progress and problems of developing polymeric drugs with the desired properties that will remain chemically stable under conditions of food pro-

cessing and preparation, digestive enzymes, microflora, etc.

The problem of the compatibility of polymeric materials with blood is an area of concern in patients with cardiovascular prosthetic devices and artificial hearts. One of the approaches to this complex area, reviewed by Platé (285), has been to immobilize antithrombogenic substances including fibrinolytic and proteolytic enzymes such as urokinase, trypsin, urease, streptokinase, α-chymotrypsin, and actynaze, as well as heparin and prostaglandin E on potenial prosthetic polymers.

IV. Structural Activity Relationships for Immobilized Drugs

An important requirement in the design of immobilized drugs and for the elucidation of drug mechanisms of action, is for the agent in question to be linked to the matrix in a biologically active conformation. The structural activity relationships are known in varying detail for most pharmacological agents prior to their use in immobilized drug studies; however, new information concerning the structure activity relationships can often be derived from careful examination of immobilized drug data.

Two detailed examples of structural activity relationships come from the work on the polymeric and solid phase immobilized forms of catecholamines and the peptide hormone insulin.

A. Catecholamines

A considerable literatures exists concerning the structural activity relationships for catecholamines [for an extensive review see Triggle and Triggle (355)]. Despite this extensive literature, several conflicting reports have appeared concerning the biological activity of covalently immobilized catecholamines linked to supports via differing positions on the catecholamine molecules. Catecholamines coupled to supports via azo bonds are reported to be agonists (16, 85, 114, 149, 156, 364, 371, 373, 375, 378, 380, 383, 384) and antagonists (44); amide linked catecholamines (through the ethanolamine side chain) are reported to be agonists (167, 204), antagonists (44), or totally inactive (406, 395). In addition, norepinephrine coupled with glutaraldehyde via the primary amine to form a Schiff's base with subsequent reduction by sodium borohydride has been reported to be an adrenergic agonist (395) and an antagonist (44).

It is known that the hydroxyl groups at the 3- and 4-ring positions are important for biological activity of catecholamines. Elimination of one or both of these hydroxyl groups substantially reduces the biological activity. Substitutions on the ethanolamine side arm can also dramatically alter the biological activity of the catecholamines; increasing the size of an alkyl substitution on the amino group of norepinephrine increases the β-adrenergic receptor activity (355). Although some large, relatively bulky alkyl substitutions allow for considerable β-receptor activity, other less bulky alkyl substitutions result in a completely inactive molecule (13). It thus becomes difficult to extrapolate an alkyl substitution to a polymer or a Sepharose or glass bead. Alkyl substitutions which maintain the ethanolamine as a secondary amine are in many cases consistent with biological activity. The claim by Weinstein et al (395) of biological activity for norepinephrine-rabbit serum albumin-Sepharose conjugates, where the amine of norepinephrine is maintained as a secondary amine, is therefore consistent with structural activity relationships for soluble catecholamines (355).

Norepinephrine coupled to a supporting matrix, utilizing dicyclohexylcarbodiimide so that an amine bond is formed, has been reported to be inactive when contamination by norepinephrine could be effectively ruled out. Amide-linked norepinephrine-glass beads were incapable of stimulating cat papillary muscles (380) and amide-linked norepinephrine-RSA-Sepharose did not stimulate cAMP formation in leukocytes, nor did the leukocytes bind to the drug conjugates (395). In addition, norepinephrine-amide-Sepharose and glass preparations do not bind tumor cells grown in culture (369).

In the azo coupling procedure, catecholamines are reacted with an aryl diazo side arm derivative chemically attached to glass, Sepharose, or soluble synthetic polymers (16, 149, 156, 364, 369, 371, 373, 375, 383, 384). It has been shown that the azo substitution on the catecholamine is at the 6-position of the catechol ring (114, 373). The structure was determined for isoproterenol, epinephrine, and norepinephrine by cleaving the azo bond linking the catecholamine to glass beads with dithionite, which yielded an amino-substituted derivative of the catecholamine (fig. 11). The position of the amino substitution was then determined by nuclear magnetic resonance spectroscopy (fig. 11) (373). Friedman and Sieber (114, and personal communication) have also shown by an alternate method that azo substitutes occur predominantly at the 6-carbon position on the catechol ring.

In reviewing known structural activity relationships, only a limited number of 6-substituted catecholamines had been synthesized. Of these, 6-methylepinephrine was reported in 1953 to possess considerable activity on a variety of test systems (130). Subsequent to the synthesis by azo bond reduction, the 6-amino-substituted catecholamines had activity (373) similar in many respects to that of the 6-methylepinephrine described (130). 6-Aminoisoproterenol, in isolated cardiac preparations and on cAMP formation in cultured tumor cells, had 0.1% of the activity of the parent isoproterenol (370). While an amino group cannot be compared to an azo substitution with extremely bulky groups, these results indicated that the catecholamines substituted at the 6-position do possess biological activity. 6-*p*-Nitrophenyl-azo-epinephrine was synthesized by diazotizing *p*-nitroaniline to epinephrine to further test the hypothesis that a 6-substituted catecholamine can be biologically active. After extensive

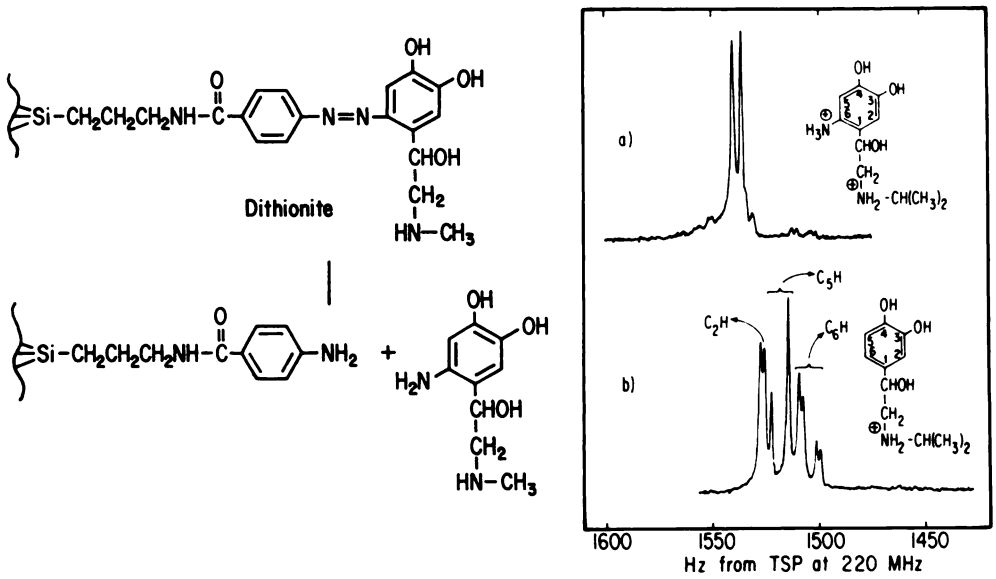

FIG. 11. (Left). Azo-bond reduction of glass bead immobilized cathecholamines (epinephrine) and the structure of 6-aminoepinephrine. Dithionite readily cleaves azo linkages to amino groups resulting in amino substituted epinephrine. Determination of the point of substitution of the new amino group gives the site of diazo substitution. (Right). Proton magnetic resonance spectrum of aromatic region of isoproterenol derivative (a) and isoproterenol (b) at 220 MHz. The concentrations were 0.1 M in D_2O-[U-^{2}H]-methanol (1:1), the chemical shifts are from TSP and the probe temperature was 40°C. The isoproterenol spectrum (b) shows the expected ABX spectrum with both ortho- and meta-coupling constants. The isoproterenol derivative (a), however, shows no ortho- or meta-coupling, which indicated that the two residual aromatic protons are para to each other, indicating the most likely site of substitution is on the 6-position of the catechol ring. Redrawn from Venter (373).

purification, the 6-(phenyl-azo)-epinephrine was more active than 6-aminoepinephrine and essentially equal in activity to epinephrine itself (176, 373). Chromatography following bioassay indicated that the azo bond remained intact during the testing procedures, indicating that the 6-phenyl-azo-catecholamines are biologically active and that the activity was not due to the formation of 6-aminoepinephrine (176, 373). Friedman and Sieber also reported on the successful synthesis of a series of 6-phenyl-azo-catecholamine derivatives that had considerable biological activity (114).

B. Insulin

Insulin contains three amino groups available for substitution and or cross-linking to a supporting matrix: the NH_2 of the A-chain amino terminal glycine and of the B-chain amino terminal phenylalanine as well as the ϵ-amino group B_{29} lysine (fig. 12). Structural activity data on insulin reviewed by Blundell et al. (27) indicates that the amino group at B_1 and B_{29} are fully accessable and more reactive than the A_1 α-amino group. The high pK of lysine makes it less reactive to electrophilic attack than the α-amino groups (27). Cuatrecasas (62) made use of this fact and demonstrated preferential coupling to B_1 or B_{29} depending upon pH. B_1 and B_{29} substitutions, while affecting immunoreactivity of insulin, only partially inhibit biological activity, whereas A_1 substitution attenuated biological potency to a greater extent (27). The majority of studies with immobilized insulin used conditions that would favor coupling through the ϵ-amino group of the B_{29} lysine (12, 24, 81, 220, 322, 347, 361). A few studies utilized only the B_1 phenylalanine (143, 248) whereas several used both the B_1 and B_{29} (61, 62, 65, 336).

Insulin contains tyrosine at A 14 and A 19 and B 16 and B 26 and histidine at B_5 and B_{10} (27). The histidine residues appear to contribute substantially to the activity of insulin because when either histidine is replaced by alanine, 95% or more biological activity is lost (393 from 27). Immobilization data is consistent with these findings. In 1972 Cuatrecasas (65) reported on the preparation of a number of insulin substituted Sepharose columns for the affinity isolation of soluble insulin receptors (fig. 2). Insulin receptors were retained on insulin affinity columns where insulin was coupled through the B_1 phenylalanine or the B_{29} lysine but not when azo coupling to histidine or histidine and tyrosine residues was used (65). More quantitative data comes from studies with soluble substituted insulin derivatives. For example, insulin coupled through B_{29} lysine to α-lactalbumin, itself highly substituted with rhodamine, retained 1.15% of its potency in stimulating glucose oxidation in fat cells and 8.3% of its binding affinity for insulin receptors (322). Hofmann et al. in 1977 (143) reported on the syntheses of biotinyl insulin, a complex between biotin and insulin, where the coupling was via the B_1 phenylalanine. The B_1-biotinyl insulin was reported to have 94% ± 9.6% of the activity of native insulin in stimulating lipogenesis in rat epididymal adipocytes (143). May et al. (220) reported on the synthesis and biological activity of NϵB_{29} biotinyl insulin where insulin was coupled to biotin via the ϵ-amino group of the B_{29} lysine. Consistent with the data of Shechter et al. (322) on B_{29} substituted insulin, the biological activity

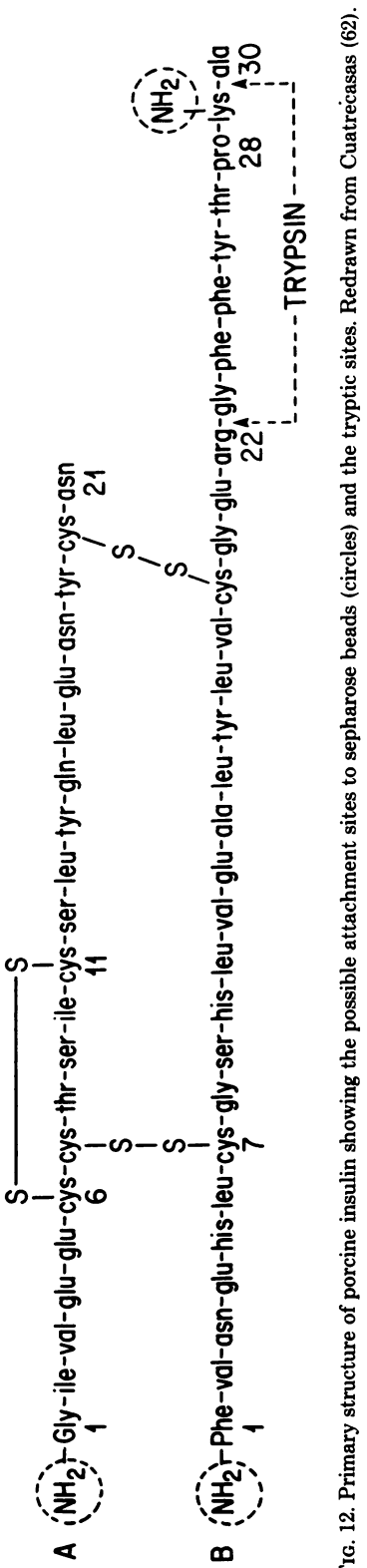

FIG. 12. Primary structure of porcine insulin showing the possible attachment sites to sepharose beads (circles) and the tryptic sites. Redrawn from Cuatrecasas (62).

of NϵB$_{29}$ biotinyl insulin was reduced to 5% of native insulin on rat epididymal fat cells (322). Specht et al. (337) coupled insulin to poly-N-vinylpyrrolidone polymers subsequent to protection of the N-terminal residues and tested the resultant complexes for biological activity. The resulting polymers of 50,000 to 60,000 MW contained an average of 180 μg of insulin per mg of polymer and retained 0.5% to 7% of the biological activity of native insulin on isolated fat cells (337).

V. Uses for Immobilized Drugs in Understanding the Sites and Mechanism of Drug and Hormone Action

A. Cellular Localization of Drug and Hormone Receptors

One of the original applications for immobilized drugs was in assessing whether the insoluble drugs exerted their actions by interacting with receptors on the plasma membrane of the target tissue (see section II and tables 1 and 2 for references). In view of the leakage problems with solid phase systems, direct confirmation of receptor location with solid support immobilized drugs does not presently appear feasible if a demonstration of biological action is the only requirement. However, the solid phase immobilized drugs do appear to be useful in demonstrating receptor locations through binding studies. Edelman and coworkers in 1971 applied the techniques of affinity chromatography to intact cells (98). With concanavalin A covalently immobilized on agarose beads, nylon fibers, and tissue culture plates, these workers bound erythrocytes via cell surface components. In 1973 Soderman et al. reported on the successful binding of isolated fat cells and fat cell ghosts to insulin immobilized on Sepharose beads (180, 336). Whereas biological activity of insulin Sepharose cannot be directly confirmed because of insulin leakage, drug release from affinity columns can, at the worst, reduce the efficiency of binding to the immobilized compound (379).

Studies by Venter et al. (369) on the binding of cultured cells to immobilized hormones showed that while binding sites for catecholamines, thyroid hormone, and ACTH appeared to be present to some degree on the cells tested, the binding was significantly greater for the target cell of the specific hormone. For example, C6 glial tumor cells bound in substantially greater numbers to isoproterenol beads than to either T_3 or ACTH beads. In addition, under conditions of maximal cell binding, a significant population of C6 cells did not bind to the columns. These same cells when challenged with soluble catecholamines did not exhibit a hormone response, suggesting a lack of cell surface receptors. Subsequent studies have demonstrated that C6 glioma cells express β-receptors in a cell cycle dependent manner, with an 80% to 90% decrease in detectable β-receptors in those cells undergoing mitosis (49). Other studies on affinity isolation of cells have involved binding of cultured hamster cells to glycolipid

glass bead complexes by Yogeeswarn et al. (405). These workers also suggested that cell binding may be cell-cycle dependent (405). Stockert et al. have reported the binding of erythrocytes to Sepharose-immobilized mammalian hepatic lectin (340). Thomas and Phillips showed the separation of human B lymphocytes on digestible immunoadsorbent columns (353). Melmon et al. reported on the ability of Sepharose drugs to remove from a given cell population only those cells that had surface receptors for histamine or norepinephrine (225, 229). Schnaar et al. (316) determined the specific adhesion of hepatocytes to immobilized N-acetyl glucosamine. These and other similar studies (7) suggest that affinity isolation of whole cells may give an indication of the cell surface receptor complement (see section II C). These techniques may have wide application to cell cloning, isolation, and characterization.

While drug leakage from solid phase immobilized drugs and hormones allows only binding events to be studied, the soluble polymeric immobilized drugs when appropriately purified can be utilized to locate exact sites of drug-receptor interactions. Biotin-complexed hormones (143, 220, 223) appear useful in this regard. Insulin and epidermal growth factor complexed to large fluorescent probes (322) have been utilized to determine the mobility and lateral diffusion coefficients for insulin and epidermal growth factor receptors in the plasma membrane of cultured cells (312).

Polymeric immobilized catecholamines (85, 149, 226, 371, 383, 384) and histamine (226) can clearly interact with cell membrane receptors in cardiac muscle (149, 371, 383, 384), human leukocytes (226), and isolated liver cells (85); further demonstrating that under controlled conditions immobilized-drug-cell membrane receptor interactions can result in the biological activation of a system.

B. Attempts to Localize Drug-Receptor Interactions to a Particular Cell Type

While studies on the affinity isolation of cells using immobilized drugs and hormones, particularly those where specific cells were isolated from mixed populations (228, 369), provide clear examples of immobilized drug action. Studies on intact tissues, though not as clear-cut, have provided some intriguing avenues for future research.

For example, the 1962 study on polymer-immobilized angiotensin suggested the cell surface as the site of action of this hormone (11). Richardson and Beaulnes extended this approach, studying both the biological effects and the histochemical location of cytochrome immobilized angiotensin. From these studies it was concluded that angiotensin must be working on the intimal endothelial cell layer of the aorta (296).

Bevan and Duckles (21) utilized norepinephrine-glass bead conjugates to assess the possible location of α-adrenergic receptors in isolated aortic strips. Norepinephrine-glass beads worked only on the intimal surface,

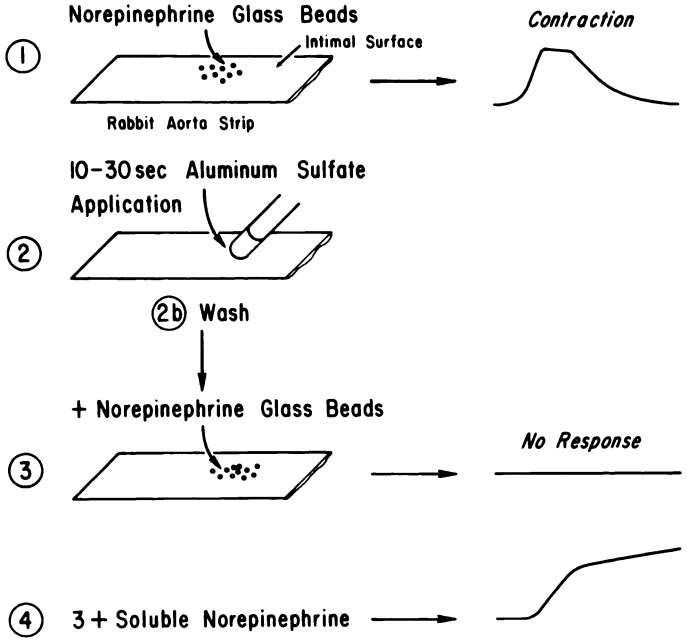

FIG. 13. Intimal cell activation of aortic strip contraction with glass bead immobilized norepinephrine. The aortic strip contractile response to glass bead-norepinephrine could be abolished by a 10 to 30-second application of aluminum sulfate to the intimal surfaces. As this figure indicates, the response to soluble norepinephrine was not effected by this treatment. Drawn from Bevan and Duckles (21).

not the adventitial surface, suggesting a role for the intimal endothelial surface of blood vessels in altering their diameter (21). A unique control experiment presented by Bevan and Duckles to aid in localizing the action of norepinephrine to the intimal surface is illustrated in figure 13 (21). The studies by Richardson and Beaulnes (296) and Bevan and Duckles (21) suggested the presence of angiotensin and α-adrenergic receptors on intimal endothelial cells of blood vessels, a presence that has subsequently been confirmed with isolated intimal endothelial cells in tissue culture (37). These studies and more recent ones by Furchgott and coworkers (50, 408) provide important new information about hormonal control of blood vessels via the intimal cells.

C. Localization of Drug Action within a Tissue

1. Propagated inotropic responses in heart muscle. In a series of studies with catecholamines immobilized on glass beads (176, 370, 371, 375, 379, 380, 381), it was demonstrated that only a minute fraction of the β-adrenergic receptors in cardiac muscle need to be stimulated by agonists in order to produce substantial positive inotropic effects (371, 372, 381). Polymeric immobilized isoproterenol, isoproterenol covalently coupled to high molecular weight amino acid copolymers (149, 370, 371, 383, 384), produced inotropic effects similar to those produced by glass bead immobilized catecholamines (371, 381). The cardiac inotropic response to immobilized and therefore diffusion-limited catecholamines was identical in character to the inotropic response obtained with isoproter-

enol free in solution (371). The positive inotropic response to isoproterenol and the immobilized derivatives has the same temporal relationship despite the differences in the amount of tissue exposed to isoproterenol in each case (149, 371, 381). When the magnitude of the catecholamine-induced positive inotropic responses are compared directly to the maximal inotropic response produced in the same muscles by paired electrical stimulation (149, 381), it becomes clear that even though in some cases as few as 0.01% of the cells in the cardiac muscle are directly exposed to isoproterenol (381), the majority of the cardiac cells in each muscle participate in the increased contractile response (149, 371, 381). These data demonstrate that while the catecholamine stimulation is limited to only relatively few cells either by diffusion and/or immobilization, all or the clear majority of the cardiac muscle cells participate in the inotropic response (149, 371, 379). These studies provided the first documentation of one apparent property of cardiac muscle, the ability to propagate an inotropic response from a site of localized catecholamine stimulation (371, 381).

Whereas the contractile responses to soluble isoproterenol and the various immobilized forms of isoproterenol are essentially identical, the cyclic nucleotide responses to these agents differed (149, 371, 381). The rapid increase in the cardiac muscle cAMP concentration in response to isoproterenol in solution was not detectable when isoproterenol immobilized on glass beads (381) or soluble polymers were used to stimulate the cardiac muscle (149).

The absence of a cAMP increase accompanying the inotropic response to isoproterenol glass beads was confirmed and extended to guinea pig papillary muscles (156). This same laboratory subsequently provided the first electrophysiological evidence for catecholamine-induced inotropic response propagation by demonstrating action potentials emanating from the site of immobilized-catecholamine application to guinea pig papillary muscles (16).

The absence of a detectable cAMP response preceeding or during the inotropic responses to immobilized catecholamines suggested that no propagation of a cAMP response occurs with the propagated inotropic response resulting from the stimulation of the superficial muscle cell layers by catecholamines (149, 371, 381).

2. Polymer immobilization of drugs to alter the diffusion coefficient of a drug. In studies on the sites of catecholamine action in cardiac muscle the extent of drug diffusion into cardiac muscle with time was compared to the kinetics of cAMP formation and to the development of the positive inotropic response to catecholamines (371). This study demonstrates one benefit of drug immobilization on soluble polymers.

Isoproterenol coupled via an azo linkage to soluble amino acid copolymers of varying molecular weights retains the ability to stimulate β-adrenergic receptors in a manner identical to native isoproterenol itself (149, 371, 383, 384). When these polymeric agents are properly purified, their biological activity is due to the covalently coupled form of isoproterenol (149, 383, 384); the high molecular weight polymeric isoproterenol derivatives were therefore utilized to study the sites of catecholamine action by exploiting the diffusion limitation of large molecules (371). Because free and polymeric immobilized isoproterenol have identical biological properties, the activity of catecholamines on the heart could be studied as a function of only the rate of access of catecholamines to cardiac muscle cells (371). The diffusion coefficient for the 13,000 MW isoproterenol polymer was 5.8×10^{-7} cm^2/sec, whereas the diffusion coefficient for isoproterenol itself was estimated to be 3.25×10^{-6} cm^2/sec (371). Assuming diffusion rates in water, it was calculated that isoproterenol would approach an equilibrium concentration throughout the muscle in 10 minutes, whereas it would require approximately 1 hour for the isoproterenol-polymer to achieve the same condition (371).

Data obtained from these studies substantiated the propagated inotropic response hypothesis for immobilized catecholamines and indicated that soluble catecholamines usually cannot stimulate muscle without some inotropic propagation occurring (371).

Altering the diffusion coefficient of drugs may also have a profound effect on the volume of drug distribution under in vivo conditions.

VI. Immobilized Enzymes as Therapeutic Agents

Enzymes immobilized on solid supports (table 1) have an exciting potential for use as therapeutic agents. Figure 14 illustrates two situations where immobilized enzymes added to an extracoporeal shunt were able to reverse experimental metabolic disorders in dogs (6, 382).

Hyperuricemia was treated with immobilized uricase (382) and experimental phenylketonuria was treated with immobilized phenylalanine ammonialyase (6). These studies show promise over conventional forms of enzyme therapy where, for example, immunological reactions to circulating enzymes limit therapy (382). In the study on immobilized uricase, antibodies formed in rabbits to the native uricase had little effect on the immobilized enzyme activity (382).

VII. Conclusions and Avenues for Future Research

While the biological effects attributed to solid phase immobilized drugs have not been substantiated, these agents have proven useful in localizing drugs to tissues and in the affinity isolation of cells and receptors. The studies with solid phase immobilized drugs led, in many cases, to investigation with drugs and hormones immobilized on soluble polymeric substances. Under carefully controlled experimental conditions, polymeric immobilized drugs can be documented to act while in the covalently coupled form. These agents have wide potential for use in basic pharmacology and physiology as well as

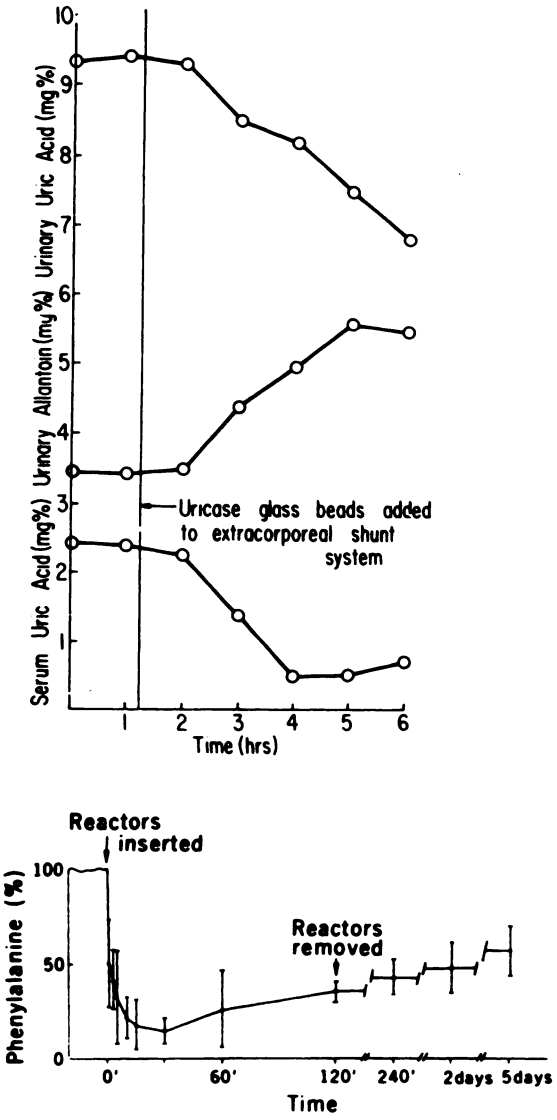

FIG. 14. Enzyme therapy with immobilized enzymes. A. Alterations in serum and urine uric acid and urine allantoin levels catalyzed by uricase glass beads in an extracorporeal shunt on an anesthetized dog. Blood was allowed to circulate through the extracorporeal shunt for 2 hours prior to the addition of uricase glass beads. Uricase glass beads (6 g) were added to the inner chamber of the cardiotomy reservoir in the extracorporeal shunt at the time indicated. Blood and urine uric acid and urine allantoin levels were determined at the indicated times. From Venter et al. (382). B. Change in blood l-phenylalanine (mean ± 95% confidence limit) with phenylalanine ammonialyase reactors in dogs having experimental phenylketonuria (PKU.) The percent of initial phenylalanine varied among the dogs from 11 to 29 mg per 100 mg of blood. Reactors were inserted into an extracorporeal shunt established between the femoral vein and femoral artery. Experimental PKU was produced by feeding the dogs 200 mg of phenylalanine per kilogram of body weight daily and 100 mg of *p*-chlorophenylalanine per kilogram of body weight every 3 days for 4 weeks. From Ambrus et al. (6).

in clinical therapeutics. Future developments will undoubtedly arise in attempts to create polymeric drugs with selective biodistributions and increased pharmacological specificity as well as in the design of more specific receptor probes. The affinity isolation of cells and receptors is also of increasing importance as the receptor field moves away from binding phenomena toward a molecular resolution of receptor structure and function.

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