Hormone–Receptor Interactions. Adrenocorticotropic Hormone Binding Site Increase in Isolated Fat Cells by Phenoxazones†

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ABSTRACT: The phenoxazone-induced rate increase of adrenocorticotropic hormone stimulated lipolysis and cAMP production in rat isolated adipocytes and fat cell "ghosts," respectively, is accompanied by a corresponding increase in the number of ACTH specific binding sites on the isolated cells. This was demonstrated with the aid of the pure, synthetic, biologically active, tritium-containing ACTH analog, [2-phenylalanine, 4-(4,5-dehydro-4,5-ditritio)norvaline]adrenocorticotropin-(1–24)-tetracosapeptide. Its specific association with the lipocytes is very rapid and easily reversible, indicating a reaction with binding sites on the plasma membrane. The relevant apparent dissociation constants are $K_{diss} \simeq 1.2 (\pm 1) \times 10^{-8}$ M for the hormone analog and $K_{diss} \simeq 1.2 (\pm 1) \times 10^{-9}$ M for the fully potent adrenocorticotropin-(1–24)-tetracosapeptide with the natural amino acid sequence. These constants remain unchanged on addition of actinomycin D and actinocin ("phenoxazones"). However, the number of specific binding sites is increased by 180% from 265 to 480 per μ^2 cell surface area by addition of 10^{-6} M phenoxazones. This corresponds very closely to the response rate increase mentioned above. The usual criteria for identifying hormone binding sites as functional "receptors" or "discriminators" (as we prefer to call them) are: (i) similarity of binding and response kinetics, (ii) similarity of the apparent dissociation constants from binding and log dose–response measurements, and (iii) correlation between the potency of hormonal analogs and their competitive inhibition characteristics for binding. All of these criteria are, individually, debatable and provide only circumstantial proof. We have an additional, strong piece of evidence to prove that the observed binding sites are indeed functional ACTH discriminators: the parallel increase of binding site number and of response rate (lipolysis, cyclase activity) caused by phenoxazones. Thus it is shown for the first time that a receptor population may contain a sizable *discriminator molecule reserve* that can be mobilized from a *dormant* to a *hormone receptive state of the molecules*. If discriminator reserve mobilization by phenoxazones were found to have its physiological correlate (not involving phenoxazones), this would indicate that hormonal regulation would not only depend on hormone blood levels, but also on the variable receptivity of target cells.

One of the fascinating areas of molecular biology is *information transduction* by the mammalian cell plasma membrane. We are trying to illuminate some of its problems by studying the one- and three-dimensional organization of information in certain polypeptide hormones as well as the chemical mechanisms of information read-out and its transduction into metabolic response by the target cells (*e.g.* Schwyzer, 1970a,b, 1973, 1974). This paper is concerned with read-out mechanisms, especially *hormone recognition* by the target cells.

Hormone–Receptors. According to current hypotheses (for an excellent review, *cf.* Rudinger *et al.*, 1972), hormones act by specific, reversible association with specialized macromolecules or macromolecular complexes of the target cells, the so-called hormone–receptors. The receptors studied hitherto appear to be proteins (*e.g.* Kono, 1969a,b, 1970; Kono and Crofford, 1972; Rodbell *et al.* 1970; Cuatrecasas, 1971, 1972; O'Brien *et al.*, 1972; Sica *et al.* 1973). Receptors for catecholamines, acetylcholine, and polypeptide hormones are located in the target cell plasma membrane (for references *cf.* Rodbell *et al.*, 1969; O'Brien *et al.* 1972; Tesser *et al.*, 1972; Birnbaumer, 1973), those for steroids in the cell interior (*cf.* Jensen and De Sombre, 1972). The hormone–receptor association (specific "binding") is probably the basic mechanism of hormone recognition by the target cell. It is also supposed to produce a "stimulus" which, in turn, evokes the first overt (metabolic, physiochemical) response or responses. This chain of events has been described in terms of the allosteric model of enzyme regulation (Monod, 1966), the hormone playing the role of an *allosteric effector*.

Adipocyte Lipolytic Receptors. Isolated cells from rat epididymal fat pads (*adipocytes, lipocytes*) respond to a variety of hormones. Among others, ACTH, glucagon, and epinephrine enhance the rates of lipolysis and cAMP production.[1] According to the "cAMP Second Messenger Hypothesis" the sole direct effect of the hormones is stimulation of adenylyl cyclase located on the cell plasma membrane inner surface. Lipolysis is supposed to be a purely cell-specific consequence of increased intracellular cAMP concentrations above background levels and to be completely detached from any direct hormone action. The postulated sequence of discrete, causal steps: hormone → cAMP → lipolysis, formally resembles the genetic "dogma," DNA → RNA → protein. Excellent reviews cover this enormous area, *e.g.* Robison *et al.* (1971), Sutherland (1972), and Birnbaumer (1973). At least in the case of adipocytes, the different hormones appear to react with different, hormone-specific, functional binding sites on the outer surface of the cell plasma membrane. The complexes between hormones and their binding sites then stimulate one and the same set of adenylyl cyclase enzyme molecules,

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[1] Abbreviations used are: ACTH, adrenocorticotropic hormone; cAMP, cyclic adenosine 3′,5′-phosphate.

enhancing their catalytic power (ATP $\rightleftharpoons$ cAMP + PP) (Bär and Hechter, 1969; Birnbaumer and Rodbell, 1969; Rodbell *et al.*, 1970; Schorr *et al.*, 1971). This and other evidence suggested the notion that hormone-receptors consist of several functional units, comparable to discriminator, transducer, and amplifier elements in technical communications systems (Hechter and Braun, 1971, and earlier references cited therein; Rodbell *et al.*, 1969; Schwyzer, 1970a). We therefore shall tentatively call the hormone-specific, functional binding sites on the outer surface of the target cell plasma membrane the *discriminators*, the enzymes or other proteins on the inside which are directly stimulated by the *hormone–discriminator complex* the *aporeceptors*, and the complex discriminator–aporeceptor systems the *holoreceptors*.

Effect of Phenoxazones on ACTH Stimulated Lipolysis. Lang and Schwyzer (1972) have recently made an observation which amounts to an apparent dissociation of the effects of ACTH on lipolysis and adenylyl cyclase activation in isolated rat epididymal fat cells: addition of 10^{-6} M actinomycin D or 10^{-6} M despeptidoactinomycin D (actinocin, devoid of antibiotic activity) almost immediately enhances the rate of ACTH induced lipolysis to ~175%, and decreases cAMP production to background levels, even in the critical interval between 0.5 and 10 min after hormone addition. Together with the results of other experiments (Lang, 1973; Sayers *et al.*, 1974) these findings suggest new *alternatives* for the current ACTH → cAMP → lipolysis model (Schwyzer, 1974).

In order to find possible explanations for these unexpected phenomena, we decided to investigate (i) the number of ACTH specific, functional binding sites (discriminators) in isolated adipocytes, (ii) some thermodynamic properties of the hormone discriminator complex *in situ*, and (iii) the possible influence of the phenoxazones actinomycin D and actinocin on these parameters (for a preliminary communication, *cf.* Schwyzer *et al.*, 1973).

Hormone–Discriminator Binding Studies. Reversible binding of hormones to cells, cell particles, membrane vesicles, or proteins has been demonstrated in many instances, *e.g.* for the insulin fat cell system (Cuatrecasas, 1971, 1972; Gammeltoft and Gliemann, 1973), the glucagon liver cell system (Marinetti *et al.*, 1972), the ACTH adrenal cell system (Hofmann *et al.*, 1970; Lefkowitz *et al.*, 1970a,b, 1971), the ACTH glucose-6-phosphate dehydrogenase system (Schwyzer and Schiller, 1969, 1971, 1973), the acetylcholine synaptic membrane protein system (Waser, 1970; O'Brien *et al.*, 1972, review; Frank and Schwyzer, 1974), the cardiac β-adrenergic receptor system (Lefkowitz, 1972), the steroid hormone receptor protein systems (Jensen and De Sombre, 1972, review). Some of the usual criteria for identification of the observed hormone binding sites with (functional) discriminators are rather equivocal. The similarity of binding and response *kinetics* is certainly a necessary, but not a sufficient condition. Similarity of the *apparent association constants* derived from binding studies on the one, and from log dose–response relationships on the other hand, are most misleading, because of the well-known phenomena of "receptor reserve" and "receptor threshold" (for a discussion with examples, *cf.* Schwyzer, 1974). Consistency between the ratios of potencies and binding constants observed for different *hormone analogs* is more convincing, although it may be argued that even nonfunctional binding sites could be structurally (*i.e.*, evolutionarily) related to the functional ones and therefore display similar binding characteristics (*e.g.* the apparently nonfunctional binding site of glucose-6-phosphate dehydrogenase specific for ACTH and the adrenal cell surface ACTH–discriminator, *cf.* Schwyzer and Schiller, 1969, 1971, 1973). Our results described here show that the two phenoxazones increase the number of adipocyte ACTH specific binding sites with $K_{ass} \simeq 10^9$ M^{-1} by ~180%. *This increase parallels that observed for ACTH induced lipolysis.* We therefore assume that these binding sites are functional, and are thus identified as discriminators. The phenoxazone phenomenon (which is specific for ACTH mediated lipolysis!) is, as far as we are aware, the first example of a "*discriminator reserve mobilization*" from a *dormant* to a *hormone receptive state* of the binding sites.

TABLE I: Amino Acid Sequences of ACTH Analogs.

1 5 10
Ser-xxx-Ser-yyy-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-
15 20 24
Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro

	xxx	yyy	
I	Phe	Nva(t_2)	= —NHCH(CH$_2$—CH(^{3}H)—CH$_2$(^{3}H))CO—
Ia	Phe	Nva	= —NHCH(CH$_2$—CH$_2$—CH$_3$)CO—
II	Tyr	Met	
III	Residues 1–6 missing from above formula		
IV	Residues 1–10 missing from above formula		

Experimental Section

[2-Phenylalanine,4-(4,5-dehydro-4,5-ditritio)norvaline]adrenocorticotropin - (1–24) - tetracosapeptide, [Phe^2,Nva(t_2)^4]-ACTH-(1–24) (I), Table I, was synthesized in our laboratory (Schwyzer and Karlaganis, 1973). Its radioactivity was determined as 7.42 Ci/mmol. The compound is a full agonist with respect to lipolysis in rat isolated adipocytes and to steroidogenesis and cAMP production in rat isolated adrenal cortex cells. However, it is about ten times less potent than the reference compound, ACTH-(1–24)-tetracosapeptide, II, prepared according to Schwyzer and Kappeler (1961) (Lang *et al.*, 1973). *Actinocin,* 2-amino-4,6-dimethylphenoxazone-(3)-1,9-dicarboxylic acid (despeptido actinomycin D), was prepared in our laboratory by R. Vogel, using essentially the method of Meienhofer (1970). *Antibiotics and other chemicals and biochemicals* were commercial products. *Isolated adipocytes (lipocytes)* were prepared from epididymal fat pads of rats weighing between 180 and 200 g according to Rodbell (1964). *Glycerol* was determined enzymatically (Wieland, 1970). *Fat cell ghosts* were prepared according to Rodbell (1967), and their *adenylyl cyclase activities* determined with a slight modification (Lang, 1973) of the method used by Ramachandran (1971).

Binding experiments were carried out after having adapted the method for measuring insulin binding by lipocytes (Cuatrecasas, 1971) to our purpose. For every *single experiment*, 0.5 ml of a pooled fat cell suspension was preincubated at 37° for 10 min with 1.5 ml of Krebs–Ringer buffer containing 3.5% of human serum albumin. Whenever phenoxazones or antibiotics were tested, they were added during the preincubation phase. Then the radioactive hormone, [Phe^2,Nva(t_2)^4]-ACTH-(1–24), was added to appropriate concentrations between about 10^{-10} and 10^{-5} M. After establishment of the hormone–discriminator complex equilibrium (2–3 min at 37°,

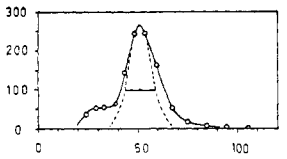

FIGURE 1: Plot of the number of cells per μ step in class j (ordinate) against class mean diameter, $(d_j + d_{j+1})/2$ (abscissa), *cf.* Table III. Gaussian distribution with standard deviation of ± 8 μ is approximated by the broken line.

see Figure 2), three samples of 0.5 ml were withdrawn. Two were used for the binding assay and one for the determination of cell dry weight (Lang and Schwyzer, 1972). The samples for the binding assay were filtered with suction over two cellulose acetate EAWP Millipore filters and washed with 3 ml of Krebs–Ringer albumin buffer. One of the wash fluids contained a high concentration ($\sim$100 μg/ml) of nonradioactive ACTH-(1–24), which displaced all the rapidly exchangeable ("specifically bound") radioactivity, whereas the slowly exchangeable ("unspecifically bound") radioactivity was retained on and by the filters. We confirmed the observation made by Cuatrecasas (1971) that the nonspecific binding (80–90%) is mainly due to adsorption on the filter material. The two filters were placed in separate vials containing Aquasol, and the radioactivity was determined by scintillation counting, using calibration curves as exemplified in Table II. The difference between the two counts ($\equiv$ "specific binding") was expressed as picomoles [Phe^2,Nva(t$_2$)^4]ACTH-(1–24) per 2 g of cells (dry weight) (each 0.5-ml sample contained between about 15 and 50 mg). One *experimental point* at a given hormone concentration is the mean of 3–4 single experiments.

The *number of cells* and their *size distribution* were determined with a Coulter Counter, Model B (Coulter Electronics Ltd., courtesy of Dr. P. Moser, Basel), providing a cell suspension containing 77.88 mg of cells (dry weight, mean of eight measurements) in 200 ml of 0.9% NaCl electrolyte and using a capillary opening 280 μ in diameter, matching switch 128, and a calibration constant 800; the results displayed in Table III and Figure 1 were obtained for a sample of 2 ml.

Results and Discussion

Adipocytes: Shape, Size, and Number. The main site of ACTH–lipocyte interaction is most probably the outer cell surface (*vide infra:* binding rate). We therefore chose to correlate our binding data with cell surface area. This required a knowledge of the shape, size, and number of adipocytes in our samples.

The shape of the isolated lipocyte is roughly spherical (Rodbell, 1964). Because of their low buoyant density and fragility, adipocytes are difficult to count by conventional methods. With the aid of a Coulter Counter, we obtained the results displayed in Table III and Figure 1. Samples of 1 mg dry weight contain about 7800 cells. These vary in diameter between around 20 and 100 μ. Their size distribution is not Gaussian: accumulations of larger and smaller particles probably reflect osmotic and mechanical damage. If all observed diameters (d) are taken into account, a mean of $\bar{d} = 49.4$ μ within 99% confidence limits for the mean of ± 0.9 μ and a standard error of ± 11 μ are obtained; using only diameters between 35 and 75 μ, the figures are: $\bar{d} = 51.5 \pm 8$ μ (99% confidence limits ± 0.7 μ). The corresponding cell surface mean areas are $\bar{f} = 7932 \pm 3689$ μ^2 (99% confidence limits ± 290 μ^2) and $\bar{f} = 8531 \pm 2654$ μ^2 (± 224 μ^2), respectively. A cell with 50-μ diameter would be expected to weigh about 130 ng (dry weight).

Our results agree well with those obtained by other methods: $\bar{d} = 40$–55 μ (di Girolamo *et al.*, 1971), and cell weights ranging from 6 to 260 ng (Hirsch and Gallian, 1968). We therefore feel justified in assuming for practical purposes a minimal *cell surface mean area of* $\bar{f} \simeq 8000$ μ^2 *per cell* (the area of a "wrinkled" surface would be larger).

[2-Phenylalanine,4-(4,5-dehydro-4,5-ditritio)norvaline]adrenocorticotropin-(1–24)-tetracosapeptide, I: Structure and Biological Activity. The radioactive ACTH analog, I, Table I, used in this study, differs in positions 2 and 4 from the well-

TABLE III: Number and Diameter (d) of Adipocytes Contained in a Representative Sample with Cell Dry Weight of 0.7788 mg.

j	Cell Diameter d_i (μ)	No. Cells Greater Than d_i	No. Cells per Class j	Cells per μ Step in Class j	$(d_i + d_{i+1})/2$
	120	0			
1			1	0.1	116
	112	1			
2			37	3.1	106
	100	38			
3			9	0.9	95
	90	47			
4			57	4.8	84
	78	104			
5			94	15.7	75
	72	198			
6			537	53.7	67
	62	735			
7			796	159.2	59.5
	57	1531			
8			1731	247.3	53.5
	50	3262			
9			981	245.3	48
	46	4243			
10			875	145.8	43
	40	5118			
11			255	63.8	38
	36	5373			
12			263	52.6	33.5
	31	5636			
13			311	51.8	28
	25	5947			
14			139	34.8	23
	21	6086			

TABLE II: Typical Calibration of [Phe^2,Nva(t$_2$)^4]ACTH-(1–24), I, by Scintillation Counting.[a]

No.	Moles/ml (x)	Cpm (y)
1	5×10^{-13}	1,300
2	1×10^{-12}	3,000
3	5×10^{-12}	12,900
4	1×10^{-11}	30,900

[a] Linear regression with least-squares method gives: $y = 3.07\ (\pm 0.2) \times 10^{15}x - 639\ (\pm 1100)$, and a correlation coefficient $r = 0.99997$.

TABLE IV: Radioactivity Specifically Bound from 5×10^{-8} M Solutions of I by Various Adipocyte Concentrations.[a]

No.	Cells (mg dry weight) per 0.5-ml sample (x)	Radioactivity Specifically Bound, Δcpm (y)
1	16.8	1700
2	21.5	2450
3	43.7	4300
4	66.5	6100
5	90.0	9000
6	103.0	9300

[a] 1 mg of cell dry weight contains about 7800 lipocytes. Linear regression with least-squares analysis gives: $y = 338 (\pm 295) + 90.26 (\pm 4.5)x$ and a correlation coefficient of $r = 0.997$. Mean specific binding: 2.52×10^6 molecules per cell, or 320 molecules per μ^2 cell surface area.

known reference peptide, II (Schwyzer and Kappeler, 1961). It was synthesized chemically from amino acids and shown to be of high purity and to contain 92% of the tritium label in the side chain of 4-norvaline; only 5% of the radioactivity resides on 6-histidine, and less than 1% on each of the other 22 amino acid residues (Schwyzer and Karlaganis, 1973). I was found to be indistinguishable from its nonradioactive counterpart, Ia, in a number of biological tests (Lang *et al.*, 1973). These observations give us confidence that the observed binding is that of peptide I (diluted, of course, with Ia), unaffected by varying, unknown amounts of more or less potent by-products. This view is also supported by the linear relationship between specific binding of I and cell concentration, as shown in Table IV.

The fact that I (like Ia) is a full ACTH agonist (lipolysis, steroidogenesis, and cAMP production in isolated cells and cell ghosts) indicates its reaction with the same set of receptors that interacts with II and with the natural hormone, ACTH-(1–39) (Lang *et al.*, 1973). Its reduced potency in lipolysis experiments reflects either an about ten times smaller apparent affinity constant towards the discriminators (K_D) or a receptor threshold mechanism in the holoreceptor. Binding competition experiments with II (Table V) indicate that the former interpretation on the basis of a reduced K_D is correct. As a consequence of reduced binding and potency, higher concentrations of I must be used, which simplifies handling and enhances accuracy.

Binding: Rate, Reversibility, and Specificity. Rapid binding rates of I by isolated fat cells and quick reversible displacement by II agree with the notion of the binding process being a reversible, cell surface phenomenon.

The results displayed in Figure 2 show that equilibrium is reached within 1–2 min at 37° after addition of I in the concentration range of 7.5×10^{-10}–3×10^{-8} M. Whereas a similar velocity was observed in the glucagon liver cell membrane system (Marinetti *et al.*, 1972), the insulin adipocyte system reacts considerably more slowly (Cuatrecasas, 1971); whether or not this might reflect a slow rate of insulin dimer to monomer dissociation (nonbinding to binding species?) is unclear. In fat cells, the two ACTH induced responses (lipolysis and adenylyl cyclase stimulation) appear simultaneously with the binding, that is, within 2 min after application of the hormone (Manganiello *et al.*, 1971; Lang and Schwyzer, 1972). However, the simultaneity is no proof of a causal relationship.

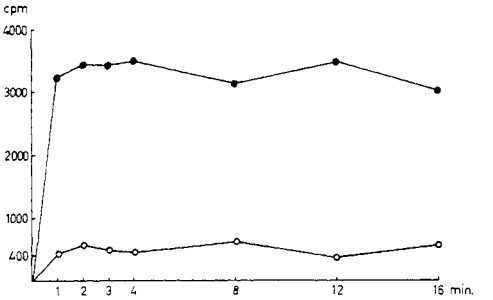

FIGURE 2: Plot of radioactivity specifically bound by equal samples of isolated adipocytes from [Phe^2,Nva(t_2)^4]ACTH-(1-24), I, solutions ((○) 3×10^{-8} and (●) 5×10^{-9} M) at 37° (ordinate) against time after mixing (abscissa).

Reversibility of binding of I is rapid and specific. Mere washing of the lipocyte–I complex on the filter with excess II displaces the "specifically bound" portion. Addition of equal concentrations of II as of I to the incubation mixtures reduces bound radioactivity to about $^1/_{20}$; $^1/_{10}$ the concentration reduces to $^1/_2$ (Table V). This is quantitatively in agreement with the observed difference between the lipolytic apparent affinity constants of I and II (*cf.* Figure 5, Table VIII, and Lang *et al.*, 1973). Further inhibitors of hormone action, like ACTH-(7–24)-octadecapeptide, III, and ACTH-(11–24)-tetradecapeptide, IV (Seelig *et al.*, 1971), also specifically displace I from its fat cell binding sites (to be published later). Contrastingly, even high concentrations of glucagon and adrenalin have no effect, confirming that these lipolytic and adenylyl cyclase activating hormones react with other discriminator molecules than ACTH. Although actinomycin D enhances the number of ACTH specific binding sites, their specificity and reversibility characteristics remain unchanged (Table V).

If the cells were incubated with I for a period of 1–2 hr, no radioactivity remained bound. However, addition of a second lot of I resulted in an unimpaired, renewed binding. This indicates that the binding sites remain hormone receptive, but lose their bound I. Whether or not this is due to proteolytic enzymes, we have not investigated. However, prolonged preincubation of the hormone alone with the Krebs–Ringer 3.5% albumin buffer at 37° for 1–2 hr inhibits its binding to the cells. This could indicate slowly reacting, but strongly binding sites present in the albumin preparation used.

Binding: Association Constants (K_{ass}) and Maximum Binding Capacity (P_{max}). Evaluation of about 70 binding experiments

TABLE V: Radioactivity Specifically Bound by Adipocytes from 10^{-8} and 10^{-9} M Solutions of I, and the Influence of Added Hormones and Actinomycin D.

No.	Conditions	% of Control
1	10^{-8} M I	≡ 100
2	$1 + 10^{-9}$ M II	54
3	$1 + 10^{-8}$ M glucagon	110
4	$1 + 10^{-8}$ M adrenaline	100
5	$1 + 5 \times 10^{-6}$ M actinomycin D	147
6	5 + 2	85
7	10^{-9} M I	≡ 100
8	$7 + 10^{-9}$ M II	5
9	$7 + 5 \times 10^{-6}$ M actinomycin D	244
10	9 + 8	11

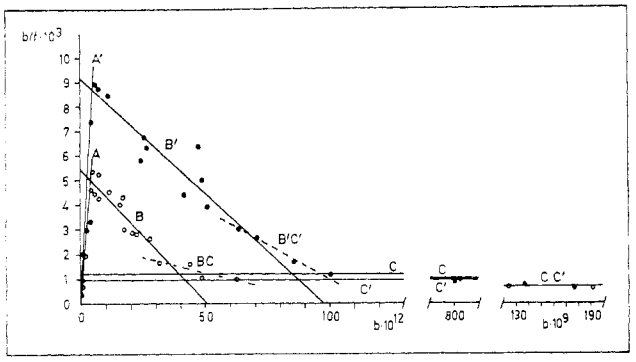

FIGURE 3: Scatchard plot of [Phe²,Nva(t₂)⁴]ACTH-(1–24), I, binding to adipocytes in the absence (○) and in the presence (●) of 5×10^{-6} M actinomycin D or actinocin: (ordinate) concentration bound over concentration free per 2 g per l. of adipocytes (b/f); (abscissa) concentration bound per 2 g per l. of adipocytes (b). Linear least-squares regression lines were derived from hormone concentrations between 0.5×10^{-10} and 5×10^{-10} (A, A'), 5×10^{-10} and 3×10^{-8} (B, B'), and 5×10^{-8} and 3×10^{-6} (C, C'); BC and B'C' were calculated from points lying between $b \times 10^{15} =$ 30–65 and 60–90, respectively ([H] $\simeq 5 \times 10^{-8}$ and 5×10^{-7}). The relevant regression lines can be described by: (B) $y = (5.45 \pm 0.27) \times 10^{-3} - (10.67 \pm 1.72) \times 10^{7} x$, $r = 0.900$; (B') $y = (9.15 \pm 0.51) \times 10^{-3} - (9.29 \pm 1.54) \times 10^{7} x$, $r = 0.918$; (C) $y = 1.2 \times 10^{-3} - 3.4 \times 10^{3} x$; (C') $y = 0.96 \times 10^{-3} - 2 \times 10^{3} x$; (BC) $y = 2.5 \times 10^{-3} - 2.5 \times 10^{7} x$; (B'C') $y = 6.5 \times 10^{-3} - 5.6 \times 10^{7} x$ wherein y is the approximated ordinate and r the correlation coefficient. B and B' extrapolate to $x = 51.1 \times 10^{-12}$ and 98.4×10^{-12} M per 2 g per liter of adipocytes ($y \equiv 0$), or 247 and 375 binding sites per μ^2 cell surface, respectively.

(without actinomycin D) by the Scatchard method (Scatchard, 1949) gave the results displayed in Figure 3. Intuitively, three regions can be distinguished. Region A, obtained with very low hormone concentrations ($<5 \times 10^{-10}$ M), is characterized by a positive slope, *i.e.*, an apparently negative association constant. We believe this region to be an artefact and to result from adsorption of small amounts of hormone (10^{-11}–10^{-10} *M*) to pipets and vessels. As a consequence the lipocytes are brought into contact with relatively much smaller hormone concentrations than initially added. Such losses are less serious at higher hormone concentrations. Region B (5×10^{-10}–3×10^{-8} M I) exhibits a normal negative slope corresponding to $K_{ass} \simeq 1 \times 10^{8}$ M^{-1}, and $P_{max} \simeq 247$ molecules/μ^2 cell surface. Evaluation of the same binding parameters using log dose–response curves (Figure 5, Table VIII) or the well-known Hill and Lineweaver–Burk equations (Fisch *et al.*, 1972) gave similar values (Table VI). Interestingly, the Hill coefficient is larger than unity, perhaps indicating a certain degree of binding cooperativity (this remains to be investigated more closely).

If we assume an ample area of 2500 Å² per binding site on the membrane surface, only 0.66% of the cell surface would be occupied by ACTH-discriminators, which also appears, *prima vista*, to be a reasonable figure.

In their studies with membrane particles of mouse adrenal tumors and iodinated ACTH, Lefkowitz *et al.* (1973) found binding sites with $K_{ass} \simeq 3 \times 10^{7}$ M^{-1}, which corresponds quite well with our figures. The few high order sites reported by those authors ($K_{ass} \simeq 9 \times 10^{11}$ M^{-1}) are not always detectable (J. Roth, personal communication). If such sites were present in lipocytes, they would have to show up in region A which, with our radioactive preparation, presents great experimental difficulties.

Region C appears at hormone concentrations above about 5×10^{-8} M, which are definitely higher than "physiological." The association constants of these binding sites are in the range of 10^{-3}–10^{-4} M, and P_{max} amounts to very roughly 10^{5} molecules/μ^2. This seems to be an impossibly large number for cell surface binding sites, because the total area would have to be completely covered by small ACTH-discriminators, each one occupying only about 60 Å². We do not know what these "very low order" sites are due to. Their characteristics are not altered by phenoxazone addition.

The Phenoxazone Effect. Table VII shows the influence of actinomycin D, of three other antibiotics, and of actinocin on the amount of I bound by lipocytes, and on the lipolysis rate produced by II. Both effects are enhanced between 1.5- and 2.0-fold by the phenoxazones, irrespective of their antibiotic properties. The other antibiotics tested influenced binding and lipolysis only slightly (Table VII, *cf.* also Lang and Schwyzer, 1972). As shown in Figure 4, the phenoxazone effect is concentration dependent. Both lipolysis and binding capacity are increasingly enhanced from 10^{-1} to 10^{2} pmol of phenoxazone per mg of cell (dry weight), where binding

TABLE VI: Association Constants, Maximal Binding Capacity, and Hill Coefficients of the [Phe²,Nva(t₂)⁴]ACTH-(1–24) Fat Cell Binding Site Interaction.[a]

	Regions			
	B	B'	C	C'
K_{ass} (M^{-1})				
Log dose[b]	7.5×10^{7}	6.8×10^{7}		
Scatchard	10.7×10^{7}	9.3×10^{7}	3×10^{3}	2×10^{3}
Hill	9.3×10^{7}	9.7×10^{7}		
Lineweaver–Burk	7.8×10^{7}	8.7×10^{7}		
Sites/μ^2				
Log dose[b]	265	510		
Scatchard	247	475	$\sim 10^{6}$	$\sim 10^{6}$
Hill	282	464		
Hill coefficient	1.36	1.32		

[a] Calculations from the data used in Figure 3 divided into two different concentration ranges (B, B' $\sim 5 \times 10^{-10}$–3×10^{-8}, and C, C' $\sim 5 \times 10^{-8}$–3×10^{-6} M; B' and C in the presence of 5×10^{-6} M actinomycin D or actinocin). Scatchard, least-squares linear regression, *cf.* Figure 3; Hill/Lineweaver–Burk, calculations by an iteration method, *cf.* Fisch *et al.* (1972). [b] *Cf.* Figure 5 and Table VIII.

TABLE VII: Influence of Antibiotics and of Actinocin on the Amount of [Phe^2,Nva(t_2)^4]ACTH-(1–24), I, Bound by Isolated Lipocytes and on the Lipolysis Rate Produced by ACTH-(1–24), II.[a]

Conditions[b]	Binding[c]	Lipolysis[c]
I or II alone	100	100
+ Puromycin	105	135
+ Chloramphenicol	121	102
+ Cycloheximide	113	93
+ Actinomycin D	182	200
+ Actinocin	186	193

[a] Representative experiments with two single batches of lipocytes each for binding and lipolysis. Evaluation of multiple experiments *cf.* Lang and Schwyzer (1972), Figure 3, and Table VI. [b] 2×10^{-8} M I for binding, 2×10^{-8} M II for lipolysis experiments. [c] Expressed in per cent of the effect obtained by either I or II alone (*Binding:* 15 femtomoles/mg of cells dry weight. Lipolysis, 2.83 nmol of glycerol produced above background in 30 min per mg of cells dry weight.)

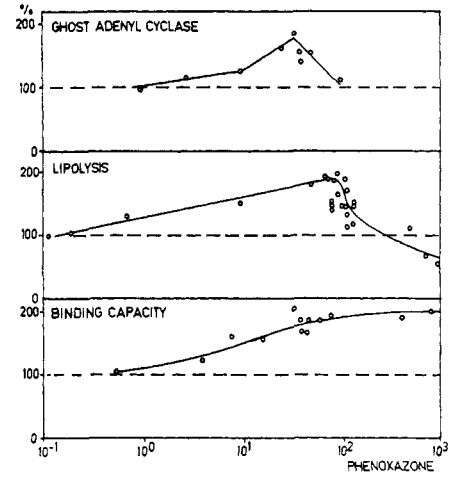

FIGURE 4: Influence of varying amounts of actinomycin D on fat cell ghosts (adenylyl cyclase activity) and on isolated adipocytes (lipolysis and [Phe^2,Nva(t_2)^4]ACTH-(1-24), I, binding). Adenylyl cyclase activity and lipolysis were stimulated by 10^{-5} and 5×10^{-8} M ACTH-(1–24), II, respectively. Experiments with varying amounts of cells or ghosts per ml suggested that not the concentration of the antibiotic is decisive, but its absolute amount (pmoles) relative to cell dry weight (mg) or ghost protein (mg), as indicated on the abscissa. The ordinate shows responsivity or occupancy in per cent of maximal responsivity (occupancy) without phenoxazone (control experiments indicated a similar behavior of actinocin).

reaches a plateau and lipolysis a maximum. Cyclase activity of fat cell ghosts is influenced in a manner similar to lipolysis. This observation also supports the assumption that phenoxazones act in the cell plasma membrane. We are presently studying possibilities for explaining the occurrence, side by side, of plateau and maxima.

The results of about 70 binding experiments in the presence of 5×10^{-6} M phenoxazone ($\sim 10^2$ pmol/mg of cells (dry weight)) are displayed in the Scatchard plot of Figure 3. Regions A′ and C′ are hardly influenced by the phenoxazones (*cf.* also Table VI). However, region B′ is shifted parallel to B in the direction of enhanced binding capacity. The association constant remains practically unaltered. P_{max} is increased by 180% from a mean of 265–483 molecules per μ^2 cell surface area (Table VI). This nicely parallels the observed influences on lipolysis (Lang and Schwyzer, 1972) and adenylyl cyclase activation of about 180 and 150%, respectively; *cf.* Table VIII.

Are the Observed Binding Sites Functional ACTH-Discriminators? There are three main reasons for believing that the binding sites of region B and B′ are indeed functional ACTH-discriminators. All three are based on the phenoxazone effect. They are: (i) the parallel increase of lipolysis rate, adenylyl cyclase activation, and binding site number, (ii) the practically unaltered association constant, $K_{ass}^{B} = (8.83 \pm 1.5) \times 10^7$ M^{-1} and $K_{ass}^{B'} = (8.63 \pm 1.3) \times 10^7$ M^{-1} (means of values in Table VI), and (iii) the unchanged specificity of binding competition (Table V). These arguments do not apply to regions A, A′ and C, C′.

If the phenoxazones are added to already lipolyzing cell suspensions stimulated by ACTH, an almost instantaneous increase of lipolysis rates—and of binding sites—is observed (Lang, 1973). This *rapid* conversion of dormant discriminators to the hormone-receptive state, for which we would like to

TABLE VIII: Hormone Concentration for Half-Maximal Effect, $[H]_{50}$, Maximal Response, R_{max}, and Maximum Binding Capacity, B_{max}, in Isolated Adipocytes or Fat Cell Ghosts.[a]

		Added Phenoxazone	
		0	5×10^{-6} M
Isolated Adipocytes			
Lipolysis by II	$[H]_{50}$[b]	9.29 ± 2.3 (−10)	2.10 ± 0.61 (−9)
	R_{max}[c]	2.54 ± 0.16	4.55 ± 0.30
Lipolysis by I	$[H]_{50}$	8.74 ± 3.3 (−9)	
	R_{max}	2.38 ± 0.26	
Binding of I[d]	$[H]_{50}$	1.34 ± 0.46 (−8)	1.48 ± 0.26 (−8)
	B_{max}[e]	27.5 ± 6.4	52.8 ± 5.2
Ghost Adenylyl Cyclase			
Activation by II	$[H]_{50}$	4.20 ± 0.76 (−7)	2.68 ± 1.28 (−7)
	R_{max}[f]	3.23 ± 0.17	4.89 ± 0.62

[a] *Cf.* Figure 5. [b] Molar concentration with exponent of ten in brackets. [c] Nanomoles of glycerol produced above background per mg of cells dry weight in 30 min. [d] $[H]_{50}$ for II-binding assumed to be ten times less. [e] Femtomolar of I per mg of cell dry weight per liter. [f] Nanomoles of cAMP produced above background per mg of ghost protein per 15 min.

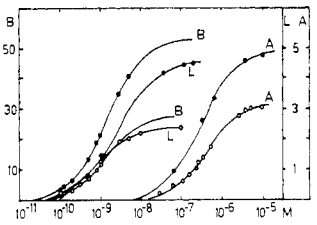

FIGURE 5: Log dose–response relationships in the systems of rat isolated adipocytes or rat fat cell ghosts plus ACTH-(1–24), II, without (○) or with (●) 5×10^{-6} M phenoxazone: (abscissa) molar concentrations of II; (ordinates) femtomolar II per mg of cells dry weight per l. (B = binding site occupation); nanomoles of glycerin produced above background per mg of cells dry weight per 30 min (L = lipolysis); nanomoles of cAMP produced above background per mg of ghost protein per 15 min (A = adenylyl cyclase activation). The individual points are means of 3–4 single experiments. Binding was calculated on the basis of the inhibition experiments of Table V, assuming a ten times larger K_{ass} for II than that observed for I. The curves were fitted to the points by a nonlinear least-squares fit to $R/R_{max} = [H]/([H] + [H]_{50})$ (Cleland, 1967), introduced into our laboratory by Dr. J.-L. Fauchère (program DOLORES). The calculated parameters are shown in Table VIII.

introduce the term *Discriminator Reserve Mobilization*, is almost certainly a direct effect of the lipid soluble phenoxazones on the cell membrane (antibiotic activity not required). The enhancement of ghost adenylyl cyclase activity also points to the cell plasma membrane as the principal site of phenoxazone action. As a working hypothesis, one could imagine mobilization as a "surfacing" of submerged discriminators, or as a conformational change of the hormone-recognizing sites.

Do these ACTH–discriminators stimulate adenylyl cyclase or other, as yet unknown, lipolytic aporeceptors? Figure 5 compares log dose–discriminator occupancy and log dose–responsivity curves for ACTH-(1–24), II. Phenoxazones enhance binding site number and the maximal responses (rates) of *both* the cellular lipolytic *and* the membrane-bound adenylyl cyclase systems. The binding curves follow closely those for lipolysis and not those for adenylyl cyclase activation. Adenylyl cyclase activation requires about two orders of magnitude higher hormone concentrations than binding. This is probably not only due to a possibly higher degree of receptor denaturation in the "ghost" preparations as compared to isolated, whole adipocytes: in the case of isolated adrenal cortex cells (no ghosts!), the same trend can be observed, because cAMP accumulation always requires between 20 and 40 times higher hormone (ACTH and analog) concentrations than steroidogenesis (Beall and Sayers, 1972; Seelig and Sayers, 1973).

At the present stage of our investigations, region B appears to represent a reasonably homogeneous discriminator population. It is impossible to distinguish between "lipolytic" and "adenylyl cyclase activating" binding sites for I with K_D's corresponding to the $[H]_{50}$'s for the two different responses (expected K_D's: 10^8 M^{-1} *vs.* 10^7–10^6 M^{-1}). Bearing in mind the possibility that the lower K_D could still be "buried" in the data lying between the regions B and C of Figure 3, we can say that our experiments do not disprove the notion of one set of discriminators operating both responses. We are performing additional experiments, especially with isolated adrenal cortex cells, in order to illuminate these questions and hypotheses.

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Ribonucleic Acid Isolated by Cesium Chloride Centrifugation†

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ABSTRACT: A quick and simple procedure based upon CsCl ultracentrifugation for the isolation of RNA from disrupted cells or subcellular fractions is described. rRNA is obtained intact and can be recovered in yields close to 100%. It is shown that the translation of rabbit globin mRNA is not impaired by exposure to this treatment. The yield of poly(A) containing globin mRNA extracted by this method and partially enriched on a poly(U)-Sepharose column was assayed in wheat germ S-30 extracts along with that extracted with phenol undergoing identical treatment. RNA from the CsCl extraction yields RNA six times more active in globin synthesis. It is observed that specific aggregation takes place during CsCl centrifugation at high gravitational fields diminishing the amount of the 16S or 18S rRNA component but not 26S or 28S component. The aggregation can be avoided by slow-speed centrifugation or reversed by heating at 60° for 30 sec. The CsCl centrifugation appears to be superior to phenol extraction in terms of simplicity, processing time, yield, intactness, and biological activity of resulting RNA. The suitability of the method for small amounts of starting material and one step separation of macromolecules from each other seem to recommend the CsCl centrifugation as a general method of isolating RNA over other methods currently in use.

The isolation of RNA from tissues, whole cells, or subcellular organelles requires a procedure in which proteins, polysaccharides and DNA are removed and in which endogenous ribonucleases are not released in an active form. Phenol extraction (Kirby, 1968) has been most generally

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