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CATECHOLAMINES AND THE KINETICS OF LIPOLYSIS IN ISOLATED RAT ADIPOCYTES

STATISTICAL ANALYSIS AND HANDLING OF DAY-TO-DAY VARIABILITY IN DOSE–RESPONSE CURVES: A GENERAL PROCEDURE FOR ASSESSING AND MANIPULATING DOSE–RESPONSE DATA

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Abstract—(1) As a first step in studying the kinetics of the lipolytic system of rat adipocytes, the day-to-day variation between dose–response curves has been analysed. (2) Methods are described for the evaluation of large quantities of data relating noradrenaline to lipolysis. (3) A 'clustering' technique is presented which can be used both to estimate and minimise differences between curves along the response axis. Criteria are outlined to determine whether clustering is appropriate. (4) Our findings indicate that the basic form of the relationship between lipolysis and noradrenaline concentration is relatively stable and consequently that the data can be utilised to study the relationship in greater detail. (5) The techniques described should be applicable to all hormone-mediated responses and may therefore provide the first step towards a meaningful analysis of the relationship between hormone concentrations and response in many systems.

The form of the relationship between the activity of an enzyme and the concentration of its effectors is recognised to be vital in the analysis of biochemical regulatory processes. For the same reasons it is necessary to evaluate the relationship between hormone concentration and the resulting physiological response which is largely dictated by the activities of multi-component enzyme systems.

Early empirical attempts to describe this relationship originated in pharmacological studies mainly of mechanical response to drugs and neurotransmitter substances [1]. Of the metabolic responses elicited by hormones, lipolysis in mammalian adipose tissue has been intensively investigated. A kinetic analysis of this system may be of particular value since considerable progress has been made in elucidating the regulation of the enzymic events involved (for reviews see Refs. 2–4).

A major obstacle in pursuing kinetic studies on many systems consisting of whole cells or tissues is the limited precision of the experimental data which can be obtained. The problem can only be partially overcome by replication because progressive changes frequently occur both within and between preparations of cells and tissues which, in our present state of knowledge, cannot be prevented.

In a large number of experiments conducted in this laboratory over an extended period, the dose–response relationships between noradrenaline and lipolysis appeared to vary, at least superficially. However, closer analysis of the results indicated that under the standardised conditions of the experiments, the systematic variation was confined almost entirely to the response-axis [5]. This analysis is extended in the present paper and exploited in such a way as to provide a composite dose–response curve, the gross regulatory features of which can be ascertained.

Theory

Deviations among dose–response curves: their evaluation and minimisation. Given two curves C_i and C_j representing rates of lipolysis (L_{ik} and L_{jk}) for varying hormone concentrations (k) in the experiments i and j, a measure of the difference between C_i and C_j is $\Sigma_k(L_{ik}-L_{jk})^2$. To make this independent of the scale of L, the difference between C_i and C_j is defined to be:

$$\frac{\Sigma_k(L_{ik}-L_{jk})^2}{\Sigma_k(L_{ik}-m_i)^2}=\frac{1}{nS_i^2}\Sigma(L_{ik}-L_{jk})^2 \qquad (1)$$

where m_i is the mean of L_{ik}, i.e. $(1/n)\,\Sigma L_{ik}$, and S_i^2 is the variance of L_{ik}, i.e. $(1/n)\,\Sigma(L_{ik}-m_i)^2$. It is shown in Appendix 1 that this difference is minimised by transforming L_{jk} to Y_{jk}, so that:

$$Y_{jk}=S_i r_{ij}\frac{(L_{jk}-m_j)}{s}+m_i$$

$$=\frac{S_i r_{ij} L_{jk}}{S_j}+m_i-\frac{S_i r_{ij} m_j}{S_j} \qquad (2)$$

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where r_{ij} is the correlation coefficient of L_{ik} and L_{jk} given by

$$r_{ij} = \frac{\Sigma(L_{ik} - m_i)(L_{jk} - m_j)}{nS_iS_j} \quad (3)$$

The minimised difference between C_i and C_j obtained using the transformation given above is called the distance, d_{ij}, and is given by:

$$d_{ij}^2 = 1 - r_{ij}^2 \quad (4)$$

The transformation presented above represents a combination of 'shift' and 'gain' which does not affect the form of the dose–response curves. More specifically, although the scale of the curves is altered, their regulatory features as measured by either the Hill coefficient (n) or the Sensitization Index (S_i) [6] remain unchanged.

Conditions under which the deviation-minimising transformation is valid. The treatment outlined above may only be applied to the experimental data if they fulfil the following criteria. (1) All curves must be of essentially the same form, (2) all discrepancies between curves must be attributable to relative displacements or expansions along the response axis; they may not differ in the direction of the response axis, and (3) the random variation associated with each dose–response curve must be uniformly distributed.

MATERIALS AND METHODS

Methods of data analysis

The programme Search. The programme determines a number of characteristic parameters of individual dose–response curves which can be used to show how they are related to one another and therefore whether the deviation-minimising transformation can be applied (see theory). The parameter values provided also yield a basis for the elimination of grossly aberrant curves from subsequent manipulations if required.

A more detailed description of the programme is provided in Appendix 3. It consists essentially of three parts. (1) Response values for each curve are first transformed so that their overall mean and standard deviation are 0 and 1, respectively. This eliminates differences attributable to a combination of 'shift' and 'gain' along the response axis. (2) The following form of the Hill equation is then fitted by a non-linearising method to each curve: $y = (Bx^N + C)/(Ex^N + 1)$. (3) Finally, the following features of the fitted sigmoid curve are evaluated: basal lipolysis, maximal lipolysis, maximum stimulation of lipolysis and the hormone concentration which evokes half-maximal stimulation of lipolysis.

The programme Cluster. This programme executes the distance-minimising transformation (described under Theory). It selects from a set of dose–response curves, the one which is most representative and applies appropriate combinations of shift and gain to the remaining to achieve the most efficient grouping (or 'clustering') about it. The programme ('Cluster') is described in Appendix 2.

In the first stage, the mean and standard deviation, m_j and S_j, are calculated for each dose–response curve and each response L_{kj} is transformed to $(L_{kj} - m_j)/S_j$. The sum of the squares of the distances (defined earlier) from one curve to all other curves is calculated for each curve and that curve C_I is found for which this total is least. The mean and standard deviation of this curve, m_I and S_I, and the correlation coefficient relating C_I to each of the remaining curves r_{Ij} is used to cluster the entire set of curves about C_I, i.e.

$$Y_{jk} = S_I r_{Ij} L_{jk} + m_I$$

(see equation 2).

A composite clustered curve C_d is obtained by calculating the mean of all derived curves at each hormone concentration. Thus, all curves are scaled to C_s and neither the final response values, nor the ratio of the maximum lipolysis to basal lipolysis is of any absolute significance. However, the underlying form of the curves is preserved as pointed out earlier.

The programme Vector. A method for determining the systematic deviation between two curves is described by Draper and Smith [7]. In order to transform dose–response data for visual scrutiny, the programme Vector was devised using a modification of these principles which was more justified for the relatively small 'sample' of curves being analysed. The mean and associated standard deviation are calculated for sets of response values obtained at each hormone concentration as in the programme Cluster. The discrepancies between individual response values and their mean are then ascribed to one of the following classes (where, for any particular hormone concentration, R_i is any one of a set of response values of which the mean and standard deviation are R_m and S.D. respectively).

(1) Positive deviations: $R_i - R_m \geq 4$ S.D.; 4 S.D. $> R_i - R_m \geq 3$ S.D.; 3 S.D. $> R_i - R_m \geq 2$ S.D.; 2 S.D. $> R_i - R_m \geq 1$ S.D.; 1 S.D. $> R_i - R_m \geq 0.5$ S.D.; ($R_i > R_m$ throughout); (2) negative deviations: $R_m - R_i \geq 4$ S.D.; 4 S.D. $> R_m - R_i \geq 3$ S.D.; 3 S.D. $> R_m - R_i \geq 2$ S.D.; 2 S.D. $> R_m - R_i \geq 1$ S.D.; 1 S.D. $> R_m - R_i \geq 0.5$ S.D. ($R_i < R_m$ throughout); or (3) the situation where $R_i - R_m > \pm 0.5$ S.D.

Data classified in this way are visually scrutinised as follows: (a) the preponderance of positive values over negative values or vice versa is determined for each curve, and (b) the maximum number of consecutive positive or negative values and their sum is determined.

A number of other quantities that are useful in detecting systematic deviations among the dose–response curves are determined as part of the programmes search and cluster. $K_{0.5}$, which is the hormone concentration yielding a half-maximal response, is determined as part of the programme Search. Σ CC is the sum of the correlation coefficients relating any curve and each of the remaining curves and is determined in the programme Cluster: it was used both to identify abberant curves and to search for a curve sub-classification.

Curve sub-classification. As stated earlier, the analysis of the dose–response curves presented in this paper, and in particular the clustering transformation, requires that the experimental curves share

the same basic form. Several methods were used to obtain evidence of heterogeneity and to provide a basis for a sub-classification of the curves. Among these was the correlation value r_{ij}, provided by the programme Cluster and several quantities yielded by the programme Search. A further method was also applied.

Each curve was considered as a single entity and its relationship with other curves characterised by its distance from them, as defined earlier. If the curves derive from the distinct populations A and B then curves associated with A would be expected to be significantly closer to each other than to those associated with B. The curves can be regarded as associated, if the distance between them is less than d_T, some chosen threshold distance. Clearly it is possible to determine whether or not there are values of d_T at which the collection of curves divided into groups within each of which $d_{ij} \leqslant d_T$.

Other related methods discussed by Dubes and Jain [8] were considered to be excessively sophisticated for the purposes of the present analysis.

Computing. The programmes Search, Cluster and Vector were written in Algol 60. They were run either directly on an ICL 4130 computer (Computing Laboratory, University College of North Wales, Bangor) or via MOD 1 (housed at the same address) link to the CDC 7600 computer at the Manchester Regional Computing Centre. Graphs were plotted using the library routine Graphplot and the plotter peripheral of the ICL 4130 device.

Experimental

General laboratory reagents were of Analar grade and were purchased from BDH Chemicals Ltd. (Poole, U.K.).

Male Wistar rats, bred in the Department from stocks which were periodically renewed from Tuck and Sons Ltd. (Rayleigh, U.K.) were fed *ad libitum* on a laboratory diet 41B (Dixon and Sons Ltd., Ware, U.K.) and used when in the weight range 170–240 g. They were killed by cervical dislocation, and distal regions of their epididymal fat pads were excised and collected in warm physiological saline.

Isolated fat cells were obtained as described by Rodbell [9] by digesting cut pieces of adipose tissue suspended in Krebs–Ringer bicarbonate buffer [10] containing 4% bovine serum albumin (Cohn fraction V; Sigma Chemical Co., London, U.K.) with bacterial collagenase (Boehringer Corp. (London) Ltd., Lewes, U.K.).

In order to determine the lipolytic activity of dispersed fat cells, polythene tubes containing 1.4 ml Krebs–Ringer bicarbonate and bovine serum albumin were briefly gassed with O_2: CO_2 (95:5), sealed and prewarmed (37°; 5 min). Immediately before introducing fat cells, noradrenaline (0.1 ml) was added in 0.9% ice-cold saline. Incubations were started by the addition of 0.5 ml of fat cell suspension (containing cells equivalent to 100–150 mg of original tissue/ml) in Krebs–Ringer Bicarbonate and albumin (4%). The tubes were then briefly gassed, resealed and returned to the shaking water bath. Experiments were carried out in a randomised block design.

After the incubation (1 hr), an aliquot (0.4 ml) of the incubation medium was carefully removed from beneath the floating fat cells and assayed for glycerol on the same day or after storage (−20°) for less than three days.

Glycerol was determined by measuring NAD^+ reduction in an incubation with glycerokinase of *Candida mycoderma* (40–80 units/mg; Sigma Chemical Co.) and glycerol-3-phosphate dehydrogenase of rabbit muscle (120–180 units/mg Sigma Chemical Co.) by the method of Chernick [11]. NADH was measured in an Eppendorf photometer with fluorescence attachment (Netheler and Hinz, Gmbh, Hamburg, West Germany) with excitation filter 316–366 nm and emission filter 405–436 nm.

Thirty-four dose–response relationships were obtained from experiments performed on seventeen days over a period of twelve months when two or more sets of data relating glycerol release to the same ten concentrations of noradrenaline (L-arterenol bitartrate; Sigma Chemical Co; 0–2 μM) were obtained. On days when more than two sets of data were obtained, the curves used in the analysis were chosen strictly at random.

RESULTS

The dose–response relationships determined between noradrenaline and lipolysis in each of thirty-four experiments are detailed in Table 1. The dose–response curve obtained from these experiments by averaging response values at each hormone concentration is presented in Fig. 2a. The appreciable rate of spontaneous lipolysis is enhanced generally some five to ten-fold when an optimum concentration of hormone is added (see Table 1 and Fig. 2a). Each of the experiments depicted in Table 1 was compared with the composite mean curve by the programme Vector which assesses inter-experimental variation (see Methods of data analysis). This analysis showed that much of the experiment-to-experiment variation is systematic in character, since for individual curves, the data are not randomly distributed about the composite mean (Fig. 1).

As indicated earlier (see Theory) a clustering transformation can be used to minimise the systematic variation among dose–response relationships if certain criteria are fulfilled. In order to determine whether the requirement is met, that differences between curves are confined to the response-axis, an analysis of the experimental results quoted in Table 1 was undertaken. A number of terms designed to characterise the dose–response curves were calculated and compared as shown in Table 2. Values for the sum of the correlation coefficients r_{ij} relating each curve to all the remaining curves were provided by the programme Cluster. The remainder were yielded by the programme Search which firstly eliminates differences of scale along the response-axis (i.e. $m_i \rightarrow 0$; $S_i \rightarrow 1$) and then determines characteristic terms including the minimum and maximum response values taken by the transformed data (min R and max R, respectively) and the half-maximally effective hormone concentration ($K_{0.5}$). Examination of the parameters which are sensitive to dose–axis variation between experiments indicated that such variation is minimal. Of particular interest are the

Table 1. Lipolysis as a function of noradrenaline concentration

Experiment	Glycerol release (μ moles/g/h) Noradrenaline(μM)									
	0	0.0078	0.0156	0.0313	0.0625	0.125	0.25	0.5	1.0	2.0
1	1.04	1.34	1.45	2.03	2.02	3.66	3.87	8 05	7.26	8 97
2	1.09	1.77	1.68	1.48	2.79	3.11	10.40	9.58	10 97	10.22
3	1.24	1.15	1.02	1.40	1.24	4.59	4.59	8.28	8.36	7.56
4	1.62	1.24	1.27	2.51	3.99	3 52	5.92	8.36	7.54	5.59
5	0.49	0.62	0.57	0.62	0.57	0 62	5.55	5.04	7.59	19.8
6	1.58	1.43	1.43	1.43	1.24	1.24	1.62	2.25	6.18	5.34
7	1.51	1.51	1.45	1.51	1.51	1.60	3.30	4.11	5.49	5.63
8	1.52	1.50	1.49	1.37	1.49	1.94	2.53	6.06	7.76	8.28
9	2.00	2.07	1.85	2 41	2.38	3.19	5.31	6 07	10.32	8.33
10	1.46	1.76	1.62	2.31	1.97	4.34	8.01	10.25	7.6	5.19
11	1.46	2.07	2.26	2.92	2.49	2.56	3.08	8.29	4.88	7 52
12	3.05	2.32	2.90	2.32	2.32	4.85	7.59	11.16	16.06	14.74
13	1.37	1.24	1.19	1.30	1.46	1.69	3.35	2.93	11.96	3.72
14	1.22	1.30	1.15	1.22	1.40	1.98	1.37	8.27	2.34	4.91
15	1.82	2.06	1.82	2.51	3.66	3.95	5.53	10.28	7.39	7.39
16	1.77	1.52	1.94	1.64	2.85	6.67	7.32	6.58	10.95	7.48
17	2.52	2.63	2.72	2.57	2.91	4.23	9.82	7.59	11.05	16 32
18	2.61	3.21	3.56	4.12	5.02	9 72	12.61	16.92	16 91	17.00
19	3.38	4.17	3.48	4.17	5.70	7.78	12.23	11.79	15.58	12.23
20	4.02	4.11	4.29	4.86	4.86	4.74	12.15	15.21	19.38	15.18
21	2.14	2.39	2.21	2.43	2.51	3.85	5 74	7.48	10.08	11.27
22	2.32	2.56	3.00	2.91	3.46	4.96	4.72	5.34	12.66	13.64
23	2.77	3.66	4.00	5.03	4.90	8.72	10.81	19.98	20.44	19.98
24	2.82	2.94	3.12	4.12	4.63	9.40	13.55	17.80	19.11	19.98
25	2.53	2.86	3.52	4.00	4.57	13.79	14.85	16 34	17.52	20.00
26	2.84	4.33	4.51	8.24	9.98	13.99	16.34	21.28	19.99	18 31
27	2.07	2.85	3.21	5.35	31.7	4.17	9.60	11.81	12.32	13.30
28	2.48	3.65	6.18	9.12	6.13	9.00	12.92	18.93	18.93	17.74
29	2.84	3.23	2.72	3.61	4.52	9.84	16.06	14.16	14.16	17.26
30	2.14	1.97	1.99	2.20	2.46	3.05	5.64	7.65	7.16	7.80
31	2.71	2.54	2.51	5.25	6.05	11.97	15.83	15 83	15.83	15.83
32	3.21	3.48	3.83	4.67	8.64	6.04	10.77	8.18	6.67	12.91
33	1.88	2.31	2.76	4.53	4.04	5.42	9.83	9.88	17.37	13.20
34	2.31	2.31	2.24	2.91	4.54	5.70	7.52	10.88	21.23	9.29

Individual results from thirty-four experiments performed as described under Materials and Methods.

Table 2. Characterisation of thirty-four sets of data relating lipolysis to noradrenaline concentration

Experiment	ΣCC	MIN	MAX	MAX-MIN	$K_{0.5}$(μM)
1	30.14	-0.851	1.628	2.479	0.265
2	29.90	-0.864	1.134	1.998	0.156
3	30.34	-0.938	1.481	2.419	0.193
4	28.57	-0.951	1.433	2.384	0.180
5	25.04	-0.518	3.275	3.793	1.23
6	25.45	-0.570	2.009	2.579	0.588
7	30.29	-0.804	1.836	2.640	0.344
8	29.44	-0.704	1.708	2.511	0.405
9	30.03	-0.752	2.004	2.765	0.431
10	26.58	-0.926	1.365	2.210	0.172
11	26.69	-0.693	1.517	2.210	0.321
12	30.45	-0.799	1.874	2.673	0.359
13	22.63	-0.535	2.035	2.570	0.654
14	21.08	-0.674	1.583	2.257	0.357
15	28.88	-0.833	1.574	2.407	0.257
16	28.46	-1.012	1.361	2.273	0.130
17	28.52	-0.739	1.776	2.515	0.279
18	30.99	-1.023	1.374	2.397	0.147
19	30.09	-0.969	1.407	2.376	0.165
20	30.30	-0.825	1.646	2.471	0 280
21	30.44	-0.797	1.879	2.676	0.362
22	27.29	-0.544	1.978	2.522	0.817
23	30.79	-0.922	1.649	2.571	0.239
24	31.05	-0.978	1.466	2.444	0.179
25	29.67	-1.056	1.266	2.322	0.124
26	29.38	-1.025	1.263	2.288	0 128
27	30.42	-0.827	1.379	3.306	0.211
28	30.16	-0.902	1.479	2.381	0.205
29	29.35	-1.028	1.275	2.303	0.128
30	30.79	-0.863	1.427	2.290	0.208
31	29.14	-1.046	1.250	2 296	0.125
32	22.73	-1.129	0.795	1.924	0.048
33	29.99	-0.774	1.844	2.618	0.367
34	27.13	-0.597	1.875	2.472	0.527
MEAN		-0.837	1.613	2.450	0.305
SD		(0.165)	(0.413)	(0.299)	(1.182)

ΣCC is the sum of the correlation coefficients relating any curve to each of the remaining curves (obtained by the programme Cluster, see Appendix); MIN, MAX are rates of lipolysis obtained in the absence of hormone and in the presence of maximally stimulatory hormone concentrations (evaluated by optimisation using the programme search; parameters C and B/E, respectively, see Appendix). $K_{0.5}$ is the concentration of noradrenaline which yields a half-maximal lipolytic response and was evaluated by optimisation (parameter $(1/E)^{1/N}$ yielded by the programme Search). These parameters were evaluated from the raw data quoted in Table 1 following their transformation to eliminate differences of scale in the direction of the response axis (i.e. mean → 0, S.D. → 1; see Theory).

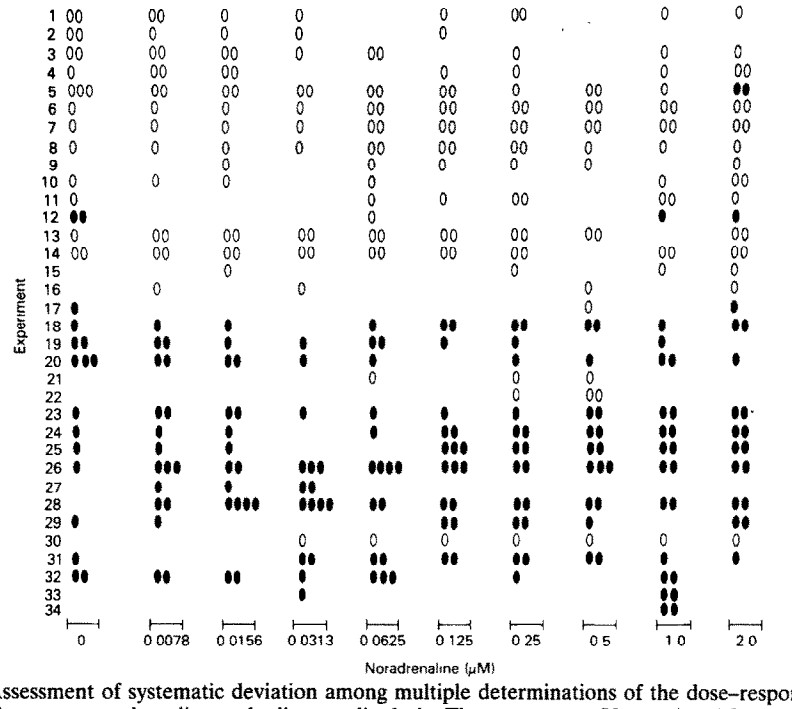

Fig. 1. Assessment of systematic deviation among multiple determinations of the dose–response relationship between noradrenaline and adipocyte lipolysis. The programme Vector (see Methods of data analysis) was used to assess discrepancies between individual response values and their means in thirty-four experiments at varying hormone concentrations. Individual data points are classified as either $R_i - R_m \pm 0.5$S.D. (no symbol); or ●●●●, 4S.D. $> R_i - R_m \geq 3$S.D.; ●●●, 3S.D. $> R_i - R_m \geq 2$S.D.; ●●, 2S.D. $> R_i - R_m \geq 1$S.D.; ●, 1S.D. $> R_i - R_m \geq 0.5$S.D. ($R_i > R_m$); and ○○○, 3S.D. $> R_m - R_i \geq 2$S.D.; ○○, 2S.D. $> R_m - R_i \geq 1$S.D.; ○, S.D. $> R_m - R_i \geq 0.5$S.D. ($R_i < R_m$).

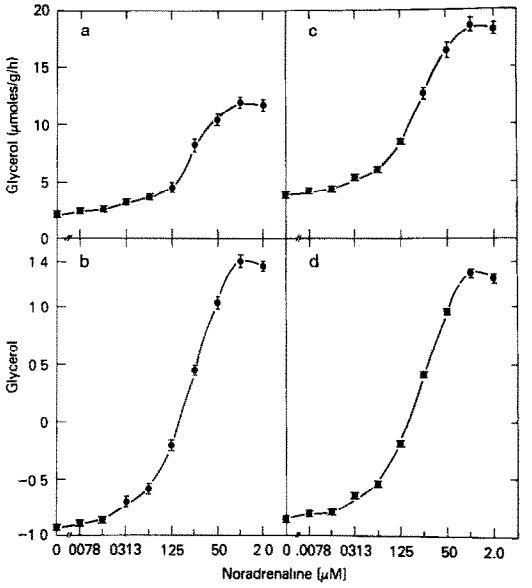

Fig. 2. The relationship between lipolytic response and L-noradrenaline. (a) Curve obtained by averaging response values observed at each hormone concentration in thirty-four determinations of the dose–response relationship (see Tables 1 and 3). (b) Curve obtained by averaging response values obtained as for (a), following transformation of the dose–response curves such that for each curve the mean and standard deviation are 0 and 1, respectively (see Table 3). (c) Composite clustered curve derived from dose–response curves obtained as for (a), scaled to the most representative curve (C_d; see Table 3). (d) Composite clustered curve derived from dose–response curves obtained as for (a), scaled to curve C_d, which is transformed so that the mean and standard deviation are 0 and 1, respectively (see Table 3).

values of $K_{0.5}$: the range of values is 0.048–1.230 μM (S.D. ± 1.182), but this is reduced to 0.240–0.654 μM (S.D. ± 0.848) if two obviously outlying curves are omitted. The anomalous position of these curves (5 and 32) is reflected in all of the comparisons shown in Table 2.

The effect of applying the programme Cluster to the thirty-four dose–response curves shown in Table 1 is summarised in Table 3 (see also Fig. 2). Since the averaged and clustered results differ in scale, the improvement in the definition of the dose–response relationship achieved in the transformation is most appropriately indicated by comparing coefficients of variation. It is clear from Table 3 that the clustering procedure improved the definition approximately three-fold. In addition, when the deviation among the clustered data was assessed by the programme Vector, it was found not to display the systematic variation shown by the original data (compare Figs. 1 and 3). It should also be noted that the deviations of the individual data from their mean as compared in Figs. 1 and 3 are measured in units of standard deviation which are considerably smaller for the clustered than for the original results.

Generally, clustered data were returned to the scale of the most representative curve C_I, but where data were to be subjected to model-fitting [10, 11] the option of clustering the results about the transform of C_I with a standardised response axis-scaling (i.e. $M_I \rightarrow 0$; $S_I \rightarrow 1$) was frequently found to be convenient (Table 3).

The thirty-four dose–response curves considered

Table 3. Evaluation of the effects of clustering on the relationship between noradrenaline concentration and lipolysis

	A				B			C			
Noradrenaline (μM)	Mean	SD	SE	CV	Mean	SD	SE	Mean	SD	SE	CV
0.00	2.11	0.76	0.13	36.0	-0.868	0.23	0.039	3.81	1.58	0.27	41.5
0.0078	2.36	0.95	0.16	40.3	-0.822	0.20	0.034	4.13	1.34	0.23	32.4
0.0156	2.50	1.19	0.20	47.6	-0.800	0.19	0.032	4.28	1.29	0.22	30.1
0.0313	3.21	1.89	0.32	58.9	-0.652	0.18	0.032	5.28	1.26	0.22	23.9
0.0625	3.59	2.05	0.35	57.1	-0.559	0.24	0.043	5.92	1.71	0.29	28.9
0.125	5.47	3.47	0.59	63.1	-0.195	0.32	0.055	8.41	2.18	0.37	25.9
0.25	8.25	4.34	0.74	52.6	0.412	0.43	0.074	12.57	2.94	0.50	23.4
0.50	10.39	4.89	0.84	47.1	0.955	0.54	0.092	16.28	3.67	0.63	22.5
1.00	12.04	5.20	0.89	43.2	1.287	0.52	0.089	18.55	3.54	0.61	19.1
2.00	11.82	5.06	0.87	42.8	1.240	0.47	0.080	18.23	3.21	0.55	17.6

Composite dose–response relationships obtained by averaging untransformed (A) and clustered (B and C) data from thirty-four experiments are compared. Clustering was performed so as to yield curves (*B*), scaled so that mean → 0 and S.D. → 1, and (*C*), scaled to the most representative curve (see text). The mean lipolysis values obtained at the noradrenaline concentrations indicated and their associated S.D. and S.E. values observed in thirty-four experiments described earlier are presented.

in this analysis (Table 1) were drawn from all experiments sharing a common design undertaken over a period of twelve months and in all cases were obtained as parts of larger experiments. No attempt was made to select subjectively from among the thirty-four curves. However, the possibility was investigated that their analysis, using the programme Select, could provide the basis of a more objective selection. Among the methods used, some were based on statistics derived from individual dose–response curves including $K_{0.5}$ and Σr^2. Another involved elimination of specific response values or complete dose–response curves if the statistics yielded by the programme vector did not fall within defined limits. In a subsequent paper [12] it is shown that the form taken by the composite dose–response curve is relatively stable to the effects of such selection procedures.

The same conclusion was drawn when the dose–response curves were scrutinised by a curve sub-

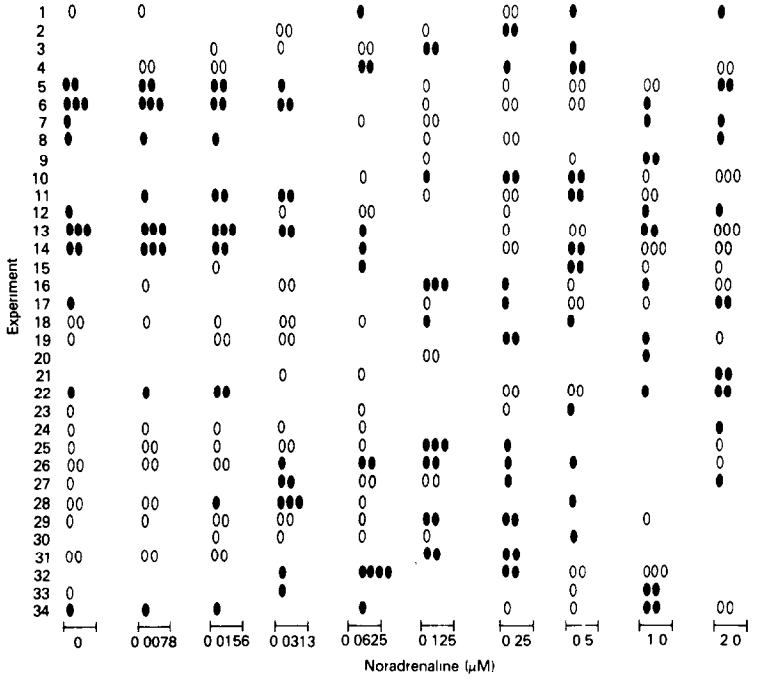

Fig. 3. Assessment of systematic deviation among multiple determinations of the dose–response relationship between noradrenaline and adipocyte lipolysis after clustering of the data. The programme Vector (see Methods of data analysis) was used to assess discrepancies between individual response values and their mean in thirty-four experiments at varying hormone concentrations after clustering. Individual data points are classified as either $R_i - R_m \pm 0.5$S.D. (no symbol); or ●●●●, 4S.D. > $R_i - R_m \geq 3$S.D.; ●●●, 3S.D. > $R_i - R_m \geq 2$S.D.; ●●: S.D. > $R_i - R_m \geq 1$S.D.; ●, 1S.D. > $R_i - R_m \geq 0.5$S.D. ($R_i > R_m$); and ○○○, 3S.D. > $R_m - R_i \geq 2$S.D.; ○○, 2S.D. > $R_m - R_i \geq 1$S.D.; ○, 1S.D. > $R_m - R_i \geq 0.5$S.D. ($R_i < R_m$).

classification method (see Methods of data analysis) which is designed to detect stratification among dose–response curves. These results are discussed further in a subsequent paper [6].

DISCUSSION

Since the shape of the dose–response curves elicited by hormones, like those yielded by any regulatory effector, is of vital importance, it is essential that precise experimental relationships are available. Although replication of experiments is valuable in this connection, great care is necessary in combining such accumulated data. Compared with the situation pertaining to individual enzymes, there is a particular difficulty which is due to the fact that the parameters by which these dose–response curves are characterised are more continuously variable. In turn, this may be so because these parameters are not products of individual molecular entities, but of systems within which such entities may vary in both abundance and spatial distribution. Thus, the day-to-day variation in replicated dose–response curves yielded by a single soluble enzyme has been dealt with relatively simply because it was possible to assume that the K_m value remained constant and the Michaelis–Menten equation was applicable [13]. However in studying the highly complex lipolytic system it was necessary at the outset to determine how much day-to-day variation occurred in its apparent affinity for hormone and to ascertain which kinetic equation was applicable.

The analysis of the relationship between lipolysis and noradrenaline concentration in this study was based on the results of thirty-four experiments to which no selection was applied. A number of parameters evaluated from these relationships indicate that they comprise a single family of curves with differences largely confined to the response-axes. Thus, the $K_{0.5}$ values quoted in Table 2 show that with two exceptions, they differ by less than five-fold. Apart from recognising that some of this discrepancy may be attributable to random error in the experimental results, the five-fold range must be seen in the context of the hundred-fold range of hormone concentrations within which the lipolytic system yields a positive response.

Application of the clustering transformation alters the scale of the response. Indeed, in this respect the scale of the clustered curve (and the selected curve C_I) is distinctly unrepresentative. Nevertheless, clustering alters the original curves only by a combination of shift and gain, neither of which alters the basic form of the relationships: in particular they do not affect the value of either the Hill coefficient (n) or the sensitization index (S_i), which measure the regulatory properties of the curves [6].

Where relative gain and relative shift are defined respectively as the gain and shift applied to an experimental curve in order to scale it to the selected (most representative) curve C_I in the programme Cluster (see Appendix), Cooper *et al.* [5] quoted the mean relative gain and mean relative shift required in order to cluster a group of forty-five noradrenaline dose–response curves as 1.37 and 1.71, respectively. The need to apply gain to dose–response curves in order to minimise their differences may be readily explained by differences in the number and size of the cells used. That it was also necessary to shift them with respect to one another is less readily accounted for in biochemical terms. Possible explanations include: (1) the hormonal environment of the fat cells in the body which may have a residual effect of considerable duration, (2) the collagenase used in the digestion of the adipose tissue, and (3) the albumin in which the cells are incubated can have a marked influence upon their metabolic activity.

The effect of transforming the dose–response relationships is most apparent where model-fitting procedures are applied [12, 14]. In this context the distribution of the error associated with the experimental results is crucial. It is also under many circumstances the aspect upon which the clustering transformation has the most striking effect. Where the composite curve represents the average of the response values obtained at each hormone concentration (Table 1) there is a sharp rise in the associated standard error (S.E.) as the response values increase. But Fig. 1. shows that the error consists of systematic variation between experiments as well as random scatter, since the positive and negative deviations associated with the individual curves are not randomly distributed.

The analysis of residual error after clustering (Fig. 2) indicates that the systematic error has been greatly reduced. The positive relationship between the standard error and the response value is decreased but is not eliminated. This almost certainly reflects the distribution of random variation among the new results. As pointed out earlier the clustering procedure is strictly applicable only if the random error is independent of the magnitude of the response (see Theory). However, although this condition is not fully met, the approximation introduced appears to be small, partly because the appreciable basal rate of lipolysis, which occurs in the absence of hormone, eliminates the need to determine very low rates of lipolysis.

Some of the methods routinely used to minimise the co-variation of the mean values obtained by experiment and their variance cannot be applied directly to data which are to be subjected to clustering. Thus if the logarithm of experimental values is taken, curves which differ in expansion and shift along the response axis no longer fulfil the requirement that they should share the same basic form (see Theory). This criterion would be met however, if the rate of basal lipolysis were first subtracted from the experimental response values. Differences of gain in the original data would then becomes differences of shift in the transformed data.

The need for such transformations differs according to the hormone- or drug-responsive system under investigation. There is little evidence that they are required for the analysis of the relationship between the concentrations of the catecholamines and the lipolytic response they elicit in fat cells, as indicated by the outcome of the optimization studies described in subsequent papers [6, 12]. Further evidence on the value of the clustering procedure in contending

with the difficulty in obtaining dose–response relationships of sufficiently precise definition for model-fitting is presented elsewhere [12].

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APPENDIX

1. *Deviations among dose–response curves: the minimising transformation*

Curve C_i is given by a set of values L_{ik}, the responses to hormone doses H_k in experiment i. Given another C_t, its distance (D_{ij}) from C_i is defined to be (see Theory):

$$D_{ij} = \frac{1}{nS_i^2}\sum_{k=1}^{n}(L_{ik} - L_{jk})^2$$

where S_i is the variance of

$$L_{ik} = \frac{1}{n}\sum_{k=1}^{n}(L_{ik} - m_i)^2$$

and m_i is the mean of

$$L_{ik} = \frac{1}{n}\sum_{k=1}^{n} L_{ik}$$

Consider transforming C_j to C_j^* by multiplying by α and adding β so that $L_{jk} = \alpha L_{jk} + \beta$ and then choosing α and β to minimise the difference between C_j^* and C_i,

$$D^* = \frac{1}{nS_i^2}\sum(L_{ik} - \alpha L_{jk} - \beta)^2 \qquad \text{(A1)}$$

The values of α and β which minimise D^* are given by

$$\frac{\delta D}{\delta \alpha} = 0$$

$$\frac{\delta D}{\delta \beta} = 0$$

$$\frac{\delta D}{\delta \alpha} = -\frac{1}{nS_i^2}\sum L_{jk}(L_{ik} - \alpha L_{jk} - \beta) \qquad \text{(A2)}$$

Equating this to 0, leads to:

$$S_iS_jr_{ij} + m_im_j - \alpha S_j^2 - \alpha m_j^2 - \beta m_j = 0 \qquad \text{(A1')}$$

Similarly, where $\delta D/\delta\beta = 0$

$$m_i - \alpha m_j - \beta = 0 \qquad \text{(A2')}$$

From (A1') and (A2'):

$$\alpha = r_{ij}\frac{S_i}{S_j}, \qquad \beta = m_i - r_{ij}m_j\frac{S_i}{S_j}$$

Substituting these values into equation (A1):

$$D^*_{\min} = 1 - r_{ij}^2$$

Therefore, where $D^{1*}_{\min}$ is defined to be the distance d_{ij} between the curves C_i and C_j:

$$d_{ij}^2 = 1 - r_{ij}^2$$

Using the values of α and β given above, it can be seen that the transform of C_j which best corresponds to c_i is given by:

$$Y_{jk} = L_{jk}r_{ij}\frac{S_i}{S_j} + m_i - r_{ij}m_j\frac{S_i}{S_j}$$

2. *The computer programme Cluster*

This programme is described briefly in the Methods section.

The set of m dose–response curves, each consisting of n values is input to store:

*Y*11, *Y*12, ...	*Y*1*n*,	response values of 1st curve
*Y*21, *Y*22, ...	*Y*2*n*,	response values of 2nd curve
..... *Yik*	*Yin*,	
..... *Yjk*	*Yjn*,	
Yml	*Ymn*,	response values of *m*th curve

where *Yik* represents the effect observed at the hormone concentration h_k in the ith experiment. Now m_i and σ_i (the mean and standard deviation of the response values, respectively) are calculated for each experiment:

$$m_i = \frac{1}{n}\sum_{k=1}^{n} Y_{ik}$$

$$\sigma^2 = \frac{1}{n}\sum_{k=1}^{n}(Y_{ik} - m_i)^2$$

The original data are then transformed so that:

$$\hat{Y}_{ik} = \frac{Y_{ik} - m_i}{\sigma_i}$$

and the transformed data, for which $m_i = 0$ and $\sigma_i = 1$, are stored. Then r_{ij}, the correlation coefficient between the ith and jth transformed curves, is calculated:

$$r_{ij} = \frac{1}{n}\sum_{k=1}^{n}\hat{Y}_{ik}\hat{I}_{jk}$$

The array:

$$r_{11}, r_{12} \ldots\ldots r_{1m}$$
$$\ldots\ldots r_{ij} \ldots\ldots$$
$$r_{m1}, r_{m2} \ldots\ldots r_{mm}$$

(in which $r_{ii} = 1$ and $r_{ij} = r_{ji}$) is then stored.

$\Sigma(cc)_i^2$ is defined as follows:

$$\Sigma(cc)_i^2 = r_{i1}^2 + r_{i2}^2 + \ldots . r_{im}^2$$

The value of i which maximises $\Sigma(cc)_i^2$ is determined and termed I. This value is the number identifying the selected

curve C_s. The transformed data are then output as the clusters $\hat{\hat{y}}$ and $\hat{\hat{\hat{y}}}$ such that:

$$\hat{\hat{Y}}_{ik} = \sigma_I r_{Ii} \hat{Y}_{ik} + m_I$$

$$\hat{\hat{\hat{Y}}} = r_{Ii} \hat{Y}_{ik}$$

The derived composite curve C_D is then obtained by calculating the mean of all the $\hat{\hat{Y}}_i$ or $\hat{\hat{\hat{Y}}}_i$ values (together with their standard deviation) at each concentration of hormone.

3. *The computer programme Search*

An outline of this programme is presented in the section on Methods of data analysis. Dose–response data Y_{ik} are read into store as described for the program Cluster. The co-ordinates Y_i^m and x_i^m are identified for which Y is maximal. Any bell-shape of 'hook' feature in the curve is then eliminated for any point x' where $x_i' > x_i^m$ by the manipulation $Y_i' = Y_i^m$.

For each curve, the mean ($\hat{m}_i$) and standard deviation ($\hat{\sigma}_i$) are rendered 0 and 1 respectively by the transformation:

$$\hat{Y}_{ik} = \frac{Y_{ik} - m_i}{\sigma_i}$$

An optimisation routine EO4CCA based on the method of Nelder and Mead [14] and available in the Nottingham Algorithms Group (NAG) Library is used to fit the following model to each transformed curve:

$$Y = \frac{BX^N + C}{EX^N + 1}$$

Goodness of fit was determined as the sum of the squared residual deviations ΣSS, to which a penalising addition was made to prevent $N < 2$.

Finally, the following terms were calculated and output:

$$C, B/E, (B/E)/C, (B/E) - C, (1/E)^{1/N}.$$