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STUDIES ON THE CONTROL OF ANTIBODY SYNTHESIS

VI. EFFECT OF ANTIGEN DOSE AND TIME AFTER IMMUNIZATION ON ANTIBODY AFFINITY AND HETEROGENEITY IN THE MOUSE

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SUMMARY

The effect of antigen dose and time after immunization on the affinity of serum anti-hapten antibody was studied in the mouse by the Farr technique and by equilibrium dialysis. A progressive increase in affinity was seen with time after immunization at all antigen dose levels. The rate of increase in affinity was faster with lower doses of antigen. However, the increase in affinity continued for a longer time in animals immunization with larger doses of antigen. Consequently, at 3 months after immunization animals injected with a larger dose of antigen had higher average affinity antibody than did animals immunized with low doses of antigen. Low affinity antibody was produced in significant amounts at all immunizing dose levels and was present throughout the course of the immune response. Certain technical problems in interpretation of equilibrium dialysis data are discussed.

INTRODUCTION

We have previously shown that individual rabbits immunized with hapten–protein conjugates produce anti-hapten antibody which is highly heterogeneous with respect to its affinity for the haptenic determinant (Eisen & Siskind, 1964; Werblin & Siskind, 1972b; Werblin *et al.*, 1973). We have also shown that the average affinity increases with time after immunization and that the rate of increase is faster with lower doses of antigen (Eisen & Siskind, 1964; Siskind, Dunn & Walker, 1968; Werblin *et al.*, 1973). Tolerance induction in neonatal rabbits was shown mainly to depress high affinity antibody synthesis with a consequent decrease in average affinity (Theis & Siskind, 1968), while passive antibody depressed predominantly low affinity antibody synthesis with a resultant increase in average affinity (Siskind *et al.*, 1968). These observations have been interpreted (Siskind & Benacerraf, 1969; Werblin & Siskind, 1972a) as consistent with a clonal selection theory of antibody synthesis in which antigen selects specific B lymphocytes to proliferate, based

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upon an interaction of the antigen (or 'processed' antigen) with cell-associated antibody present on the surface of the B lymphocytes. The cell-associated antibody is presumed to be identical in binding properties to the antibody secreted by that cell, and its progeny, after stimulation by antigen. An increase in affinity of antibody with time after immunization has been observed by a number of other workers in several different species and with a variety of antigens and immunization procedures (Fujio & Karush, 1966; Grey, 1964; Fazekas de St Groth and Webster, 1966; Klinman *et al.*, 1966; Parker, Godt & Johnson, 1967; Goidl *et al.*, 1968; Andersson, 1970; Sarvas and Mäkelä, 1970; Lamelin & Paul, 1971; Davie & Paul, 1972; Miller & Segre, 1972; Petty, Steward & Scothill, 1972; Huchet & Feldmann, 1973; Möller, Bullock & Mäkelä, 1973).

The mouse is one of the most frequently used animals for immunological research. An increase in avidity of antibody produced by mice with time after immunization has been reported by a number of workers (Andersson, 1970; Miller & Segre, 1972; Petty *et al.*, 1972; Huchet & Feldmann, 1973; Möller *et al.*, 1973). Most of these studies were carried out using indirect methods at the cellular level. We have, therefore, carried out a formal study of the affinity of serum antibody in the mouse, from 10 days to 3 months after immunization using a wide range of antigen doses. The affinity measurements reported in the present paper were obtained by the Farr technique and by equilibrium dialysis. The data are in general comparable to that previously obtained by this laboratory in studies on rabbits (Eisen & Siskind, 1964; Siskind *et al.*, 1968; Werblin & Siskind, 1972a; Werblin *et al.*, 1973) and by other workers studying mice (Andersson, 1970; Miller & Segre, 1972; Petty *et al.*, 1972; Huchet and Feldmann, 1973; Möller, Bullock & Mäkelä, 1973). However, the present studies indicate that, while initially selection for high affinity antibody synthesis proceeds more rapidly after lower doses of antigen, the increase in affinity continues for a longer time after higher doses of antigen. Consequently, late after immunization higher affinity antibody is observed after immunization with larger doses of antigen.

MATERIALS AND METHODS

Animals

Six to 8-week-old Swiss–Webster mice were used.

Antigen preparations

Dinitrophenylated (DNP) proteins were prepared essentially as described by Eisen, Belman & Carsten (1953) by reacting human serum albumin (HSA) or bovine gamma-globulin (BGG; Pentex, Kankakee, Illinois) with 1-fluoro-2,4 dinitrobenzene (DNFB; Eastman Organic Chemicals, Rochester, New York) at room temperature under alkaline conditions. The products were purified by acid precipitation and extensive dialysis. The concentration of the hapten-conjugated protein was determined by drying a known volume to constant weight at 95°C. The BGG derivative had approximately fifty DNP groups per molecule of protein by spectral analysis.

Hapten preparation

Dinitrophenylated ε-amino-caproic acid (DNP–EACA) was prepared by the reaction of DNFB with ε-amino-*n*-caproic acid (EACA; Sigma Chemical Company, St. Louis,

Missouri) under alkaline conditions. Tritiated DNP–EACA was prepared by the reaction of ^{3}H-labelled DNFB (Amersham Searle, Arlington Heights, Illinois) with EACA. The detailed procedures for both preparations have been described previously (Werblin & Siskind, 1972b).

Immunization procedures

Mice were immunized with a single intraperitoneal injection (0·5 ml) of the indicated amount of DNP–BGG emulsified in complete Freund's complete adjuvant (FCA) containing 0·5 mg/ml of killed *Mycobacteria butyricum* in the final emulsion and were bled at the times indicated. Rabbits were immunized with 0·5 mg mouse gamma-globulin (MGG) in FCA; were bled to death 6 weeks later; and the serum was pooled. A total volume of 2·5 ml of emulsion was injected divided among the four foot pads and subcutaneously into the back of the neck.

Preparation of the immunoadsorbents

Protein was coupled to bromoacetyl cellulose (BAC; Miles Laboratories, Kankakee, Illinois) according to the procedure of Robbins, Haimovich & Sela (1967) to prepare two immunoadsorbents. The first (MGG–BAC) was used for the purification of rabbit anti-mouse gamma-globulin. The second immunoadsorbent (DNP–HSA–BAC) was used for the immunoassay of mouse anti-DNP antibody concentration. The immunoadsorbents were washed serially with 0·15 M NaCl, 8 M urea, proprionic acid (pH 2·8) and 0·15 M sodium phosphate buffer (pH 7·4).

Preparation of radioiodinated rabbit anti-mouse gamma-globulin

Rabbit anti-MGG was purified by use of MGG–BAC according to the method of Robbins *et al.* (1967). The anti-MGG was eluted from the immunoadsorbent by use of proprionic acid (pH 2·8). Purified anti-MGG was iodinated with ^{125}I by the chloramine T method as described by Sonoda & Schlamowitz (1970). The specific activity of the product was approximately 2×10^{6} counts/min/μg protein.

Immunoassay of mouse anti-DNP antibody

The concentration of mouse anti-DNP antibody was assayed by use of DNP–HSA–BAC. The procedure was a modification of that described by Klinman & Taylor (1969). All dilutions and washings were made with 1% HSA. Appropriately diluted serum was added to 30 μg immunoadsorbent in a total volume of 0·4 ml and was incubated at room temperature for 30 min. The mixture was centrifuged, the supernate was removed by suction and the immunoadsorbent was washed with 0·6 ml of 1% HSA. 0·04 μg of ^{125}I-labelled anti-MGG in 0·4 ml of diluent was added and the mixture was incubated at room temperature for 30 min with occasional mixing. The immunoadsorbent was washed once by centrifugation, was resuspended in 1% HSA, collected on a millipore filter, and washed (on the filter) with approximately 3 ml of 1% HSA. The millipore filter was counted in a gamma counter. In each experiment a standard curve was constructed using a single pool of mouse anti-DNP antibody whose concentration had been previously established by saturation with hapten in equilibrium dialysis (Werblin & Siskind, 1972b). Controls using normal mouse serum were included in each run to correct for nonspecific binding of the anti-MGG.

Measurement of antibody affinity by the Farr technique

Assays of anti-DNP antibody affinity were carried out, in some cases, by the Farr technique (Farr, 1958; Stupp, Yoshida & Paul, 1969; Werblin *et al.*, 1973) using ^{3}H-labelled DNP–EACA as ligand. Bound and free hapten were separated by precipitation at 50% cold saturated $(NH_4)_2SO_4$. The methods for carrying out the Farr assays, and the procedures for computation of affinities have been described in detail in previous publications (Werblin *et al.*, 1973). Affinity measurements were carried out at 0·4 μg antibody/ml using ten different free hapten concentrations ranging from $2{\cdot}6 \times 10^{-11}$ to 8×10^{-10} M. The data were plotted according to the method of Sips (1948). 'Average' association constants ($K_{I\ 10\%}$) were calculated from the binding data falling in the range between 0·1 and 10% of antibody sites occupied by hapten. A straight line was fitted to these data points by linear regression analysis and the average affinity (association constant) was expressed as the reciprocal of the free hapten concentration at which this line indicates 50% of the antibody sites occupied by hapten. Restriction of the data used for calculation of the association constant to a clearly defined portion of the binding curve tends to eliminate uncertainties caused by the non-linearity of the binding curves, and to make the data obtained on different samples more comparable. This procedure for expressing average affinity emphasizes the highest affinity antibody subpopulations present in the sample. In the present paper data are presented as the free energy change ($\Delta F^\circ_{I\ 10\%}$) which is calculated from the association constant using the relationship:

$$\Delta F^\circ = -RT \ln K$$

where R is the gas constant and T is the absolute temperature.

Measurement of antibody affinity by equilibrium dialysis

Antibody affinity was also determined by equilibrium dialysis using ^{3}H-labelled DNP–EACA as ligand by methods previously described in detail (Werblin & Siskind, 1972b). Measurements were carried out on globulin fractions of antisera prepared by precipitation twice at 50% saturated $(NH_4)_2SO_4$. After the antibody concentration was determined by immunoassay the samples were diluted to an antibody concentration of 0·5 μg/ml and the binding curve was obtained using twenty points ranging from 4×10^{-13} to 8×10^{-7} M free hapten. The data were plotted according to the procedure of Sips (1948) and the average affinity was expressed as $\Delta F^\circ_{I\ 10\%}$ and as $\Delta F^\circ_{I\ 25\%}$ calculated as described above and in detail by Werblin & Siskind (1972b). Distributions of affinities were computed by a curve fitting procedure (Werblin & Siskind, 1972b) using a series of Fortran computer programs written by Werblin (1972).

Radioactivity counting

Samples from Farr and equilibrium dialysis were counted in 5 ml Aquasol (New England Nuclear, Boston, Massachusetts) using an ambient temperature Beckman Scintillation Spectrometer. Each sample was counted sufficiently long so as to accumulate enough counts to give a percent standard error of counting not exceeding ± 3 per cent. Samples were screened for quenching by use of an external standard and no significant quenching was observed in the studies reported here. ^{125}I was counted in a Nuclear Chicago gamma counter.

RESULTS

Effect of antigen dose and time after immunization on antibody concentration

Groups of mice were immunized with 10, 30, 300 and 1000 μg DNP–BGG in FCA. Each group of mice was bled 10 days, 3 weeks, 6 weeks and 12 weeks later. After the 12-week bleeding the animals were boosted with 500 μg DNP–BGG intraperitonealy and were bled 1 week later. As shown by Table 1, the 3-week bleeding had the highest concentration of anti-DNP antibody regardless the dose of antigen used for immunization. Mice immunized with 100 μg and 300 μg tended to have the highest concentrations of anti-DNP antibody.

TABLE 1. Effect of antigen dose and time after immunization on the concentration of anti-DNP antibody formed by mice immunized with DNP–BGG *

Antigen dose (μg)	Anti-DNP antibody concentration (μg/ml)				
	10 days	3 weeks	6 weeks	12 weeks	Post-boost†
10	259± 34	218± 32	67±32	24± 6	223± 30
30	319±130	579±130	167±57	66±20	437±111
100	370± 68	608±190	234±65	68±17	428± 97
300	353±120	728±140	248±40	66±15	294± 35
1000	402±170	529±160	109±22	98±32	567±197

* Groups of mice were immunized intraperitoneally with varying doses of DNP-BGG in FCA and were bled serially at the times indicated. Antibody concentration was determined by use of DNP–HSA–BAC immunoadsorbent. Data are presented as mean ± standard error of the mean for groups of five mice.

† Animals were boosted with 500 μg DNP–BGG intraperitoneally 1 day after the 12-week bleeding and were bled 7 days after boosting.

TABLE 2. Effect of antigen dose and time after immunization on the affinity of the anti-DNP antibody formed by mice immunized with DNP–BGG*

Antigen dose (μg)	Anti-DNP antibody affinity ($-\Delta F^2_{I\ 10\%}$; kcal/mole)				
	10 days	3 weeks	6 weeks	12 weeks	Post-boost†
10	9·13±0·09	10·20±0·16	10·56±0·16	10·26±0·15	10·47±0·25
30	8·29±1·14	10·01±0·24	10·27±0·18	10·19±0·11	10·44±0·12
100	8·43±0·50	9·31±0·21	10·27±0·12	10·86±0·18	10·45±0·35
300	8·09±0·54	9·56±0·08	10·00±0·37	11·16±0·18	10·63±0·42
1000	7·95±0·33	9·52±0·35	10·28±0·31	10·73±0·34	10·81±0·24

* Groups of mice were immunized intraperitoneally with varying doses of DNP–BGG in FCA and were bled serially at the times indicated. The animals are the same as those presented in Table 1. Average affinities were determined by the Farr technique at 20°C using DNP–EACA as ligand. The data are presented as mean ± standard error of the mean for groups of five mice.

† Animals were boosted with 500 μg DNP–BGG intraperitoneally 1 day after the 12-week bleeding and were bled 7 days after boosting.

Boosting resulted in a prompt increase in antibody concentrations to levels approximating the peak observed during the primary response.

The effect of antigen dose and time after immunization on antibody affinity

The affinity of the anti-DNP antibody measured by the Farr technique is presented in Table 2. An increase in affinity with time after immunization is seen in all groups. The rate of increase in affinity is initially greater in mice immunized with lower doses (10 and 30 μg) of antigen. Thus, at 10 days and at 3 weeks after immunization there is a clear relationship between antigen dose and average affinity. The lower the antigen dose, the higher the affinity. However, at 6 weeks all groups have the same average affinity. Animals immunized with 10 and 30 μg of antigen have reached their peak affinity by 6 weeks. These animals show no further change in affinity at 12 weeks or after boosting. Animals immunized with 100 to 1000 μg DNP–BGG continue to show an increase in affinity between 6 and 12 weeks. Consequently, groups immunized with higher doses of antigen have anti-hapten antibodies at 12 weeks which are, in fact, significantly higher in affinity than those formed by animals immunized with 10 or 30 μg of antigen. (Student's t-test: $P<0{\cdot}025$ for all comparisons of 10 and 30 μg groups with 100, 300 and 1,000 μg groups except for comparison of the 10 and 1,000 μg groups where $P = 0{\cdot}1$, and of the 30 and 1000 μg groups where $P = 0{\cdot}05$.)

TABLE 3. Effect of antigen dose and time after immunization on the affinity of the anti-DNP antibody formed by individual mice and studied by equilibrium dialysis *

Antigen dose (μg)	Time after immunization: 3 weeks $-\Delta F^{\circ}_{I\ 10\%}/-\Delta F^{\circ}_{I\ 25\%}$ (kcal/mole)	6 weeks $-\Delta F^{\circ}_{I\ 10\%}/-\Delta F^{\circ}_{I\ 25\%}$ (kcal/mole)	12 weeks $-\Delta F^{\circ}_{I\ 10\%}/-\Delta F^{\circ}_{I\ 25\%}$ (kcal/mole)
30	9·19/8·84	9·65/9·62	10·50/9·74
100	8·54/8·54	9·01/9·02	10·37/9·49

* Two individual mice were immunized with the dose of DNP–BGG indicated intraperitoneally in FCA and were bled serially as indicated. Antibody concentration was determined by use of DNP–HSA–BAC immunoadsorbent and the affinity was determined by equilibrium dialysis at 2°C using DNP–EACA as ligand. Affinities are expressed as both $\Delta F^{\circ}_{I\ 10\%}$ and $\Delta F^{\circ}_{I\ 25\%}$ which are calculated from binding data falling between 0·1 and 10% or 25% respectively of the antibody sites occupied by hapten.

Effect of antigen dose and time after immunization on antibody affinity measured by equilibrium dialysis

As discussed in a separate paper (Kim & Siskind, 1974) the Farr technique tends mainly to detect relatively high affinity antibodies. Equilibrium dialysis, while far more time consuming, gives a more precise description of the binding properties of the antibody. Therefore, samples obtained from one animal in each group were studied by equilibrium dialysis. The distributions of affinities in the samples were calculated. The progressive shift towards production of increased amounts of high affinity antibodies can be readily seen. The persistence of synthesis, in all animals, of large amounts of low affinity antibodies is

also apparent. The affinity distribution only approaches a symmetrical distribution early after immunization. Thereafter, it is highly skewed, with, at times, a tendency towards bimodality. It should be especially noted that the increase in average affinity is due mainly to increased production of a relatively small subpopulation of high affinity antibodies. Most of the antibodies present throughout the course of the response are of low affinity. 'Average' affinities calculated using either the initial 10% or 25% of the binding curve are presented for two representative animals in Table 3. The value calculated from the initial 10% of the curve is often significantly higher than that calculated from 25% of the binding curve. This is especially true late in the immune response when a small subpopulation of high affinity antibody is present (Fig. 1). The increase in affinity of mouse antibody with time after immunization is more apparent when $\Delta F^{\circ}_{I\ 10\%}$ rather than when $\Delta F^{\circ}_{I\ 25\%}$ is used to express antibody affinity.

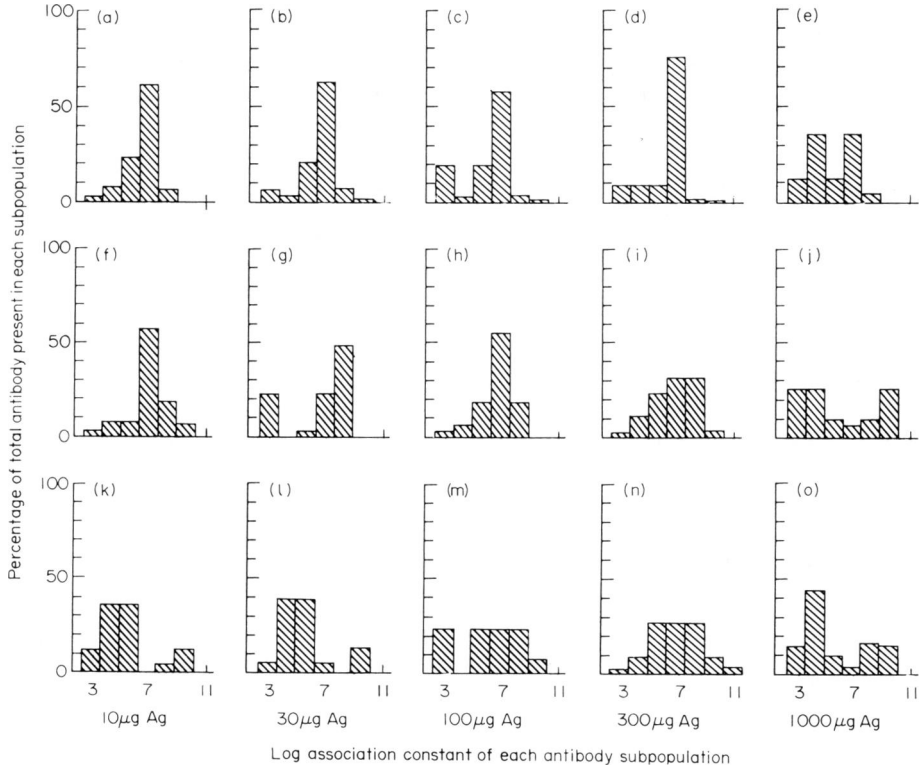

FIG. 1. The distribution of affinities of antibodies in individual mice immunized with various doses of DNP–BGG in FCA and bled at (a)–(e) 3, (f)–(j) 6 and (k)–(o) 12 weeks after immunization. The distributions were computed from equilibrium dialysis data using DNP–EACA as ligand.

DISCUSSION

A progressive increase in the affinity of serum antibody was observed in mice immunized with DNP–BGG in CFA. The rate of increase in affinity was more rapid with lower doses

of antigen. As a consequence, at 10 days and at 3 weeks after immunization animals which received suboptimal doses of antigen with regard to the amount of antibody produced, had the highest affinity antibody. Thus, animals immunized with low doses of antigen appear to synthesize less low affinity antibodies than do animals immunized with higher doses. However, selection for high affinity antibody synthesis appears to continue longer in animals immunized with higher doses of antigen. As a result, animals immunized with higher doses of antigen actually have the highest affinity serum antibody at 12 weeks after immunization.

The data are generally consistent with a clonal selection theory according to which B lymphocytes are stimulated to proliferate by interaction of antigen with 'cell-associated antibody'. As the antigen concentration decreases with time after immunization a type of microevolutionary process would be expected to take place with high affinity cells coming to predominate in the population. The selection of high affinity antibody-producing cells would thus be expected to proceed more rapidly after lower doses of antigen. It may be assumed that antigen persists longer in animals initially receiving a larger dose. This would result in selection for high affinity antibody-forming cells taking place over a longer period of time with ultimately higher average affinity antibody being produced by these animals. This is consistent with the data reported in the present paper. Recent studies using rabbits have suggested that selection so that a large fraction of the total antibody comes to lie in a subpopulation of high affinity and restricted heterogeneity may occur more often after immunization with higher doses of antigen (Werblin *et al.*, 1973). A tendency was also noted (Werblin *et al.*, 1973) for the average affinity of antibody present in rabbits very late after immunization (3–12 months) to be slightly higher in animals immunized with high doses of antigen (5–50 mg). When only the highest affinity subpopulations are examined all groups of rabbits appear to have antibody of very similar high affinity at 3–12 months after immunization (Werblin *et al.*, 1973). Thus, in both rabbits and mice it would appear that all animals eventually synthesize antibody of fairly similar high affinity regardless of antigen dose. A low dose of antigen favors rapid early selection for high affinity antibody synthesis, while a large dose of antigen leads to slower selection over a longer period of time. Under the latter conditions the high affinity subpopulations often come to constitute a larger fraction of the total antibody present than occurs after immunization with low doses of antigen. It must be noted that in all of these studies antigen is present as an emulsion in CFA. Thus, a depot of antigen is established in the animal. With other immunizing conditions different kinetics of affinity maturation may well occur.

It should be noted that significant amounts of low affinity antibodies are actually synthesized by all animals regardless of antigen dose. The differences between different groups is mainly the result of differences in the kinetics of selection of high affinity subpopulations. This is similar to what we have previously reported for rabbits (Eisen & Siskind, 1964; Siskind *et al.*, 1968; Werblin & Siskind, 1972a; Werblin *et al.*, 1973). However, with our immunizing conditions the high affinity subpopulations synthesized by mice rarely exceed 20% of the total anti-hapten antibody. Care must be taken if this relatively small amount of high affinity antibody is to be detected in the presence of large amounts of low affinity antibodies.

The best way to analyse the data is clearly by the approximation procedure which we previously described (Werblin & Siskind, 1972b). When analysed in this manner, the appearance of high affinity subpopulations with time after immunization is very apparent. If one attempts to calculate average affinities directly from a Sips' plot it is important to examine

independently that portion of the data obtained at very low free hapten concentration. Affinity values calculated from the initial 10% of the binding curve (from 0·1 to 10% of the antibody binding sites occupied by hapten) reflect changes in the high affinity subpopulations. When one uses data ranging over a larger portion of the binding curve to calculate an average affinity small changes in the high affinity subpopulations may be obscured by the large amount of relatively low affinity antibody present.

The fact that different values are obtained for the 'average' affinity when different portions of the data are analysed using a Sips' plot indicates that the antibodies are not distributed, with respect to affinity, in a symmetrical manner about some mean value. If a symmetrical (normal) distribution of affinities were present binding data would be linear when plotted in the Sips' manner and the same value for the 'average' association constant would be obtained regardless of what portion of the data was used for the calculations. The results presented emphasize the value of a detailed analysis of binding curves rather than merely a simple statement of 'average' affinity. If for the sake of simplicity sera are to be compared with regard to 'average' affinity it is crucial that the data for the different samples are strictly comparable with regard to the regions of the binding curve used for the calculations.

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