(from methyl-), or $n_O = 24$; n_O is 2 (from progesterone) plus 1 (from acetate) plus 1 (from hydroxy-), or $n_O = 4$; $R = 4$; and Δ is 3 (from progesterone) plus 1 (from acetate), or $\Delta = 4$.

$$T = 2 + 3(24) + 4 - 2(4 + 4) = 62.$$

2) If the systematic name, 2-chloro-10-(3-dimethylaminopropyl)-phenothiazine, is given, the total can be computed easily by adding increments to the fundamental phenothiazine ring system as follows: $n_O = 12 + 2 + 3 = 17$; $n_N = 1 + 1 = 2$; $n_S = 1$; $R = 3$; and $\Delta = 6$. The total is 40, the same as that of $C_{17}H_{19}ClN_2S$, the molecular formula of the compound.

The preceding methods of checking atom-totals are especially useful in the editing of technical reports and the indexing of chemical compounds according to their molecular formulas. The examples show that time might possibly be saved and that the possibility of error might be reduced for complex molecular structures. A few glances at a molecule's structure provide all information necessary for computing the molecule's total number of atoms.

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Increased Incidence of Anencephalus and Spina Bifida in Siblings of Affected Cases

Abstract. In 139 families having a child born with anencephalus or spina bifida, 10 of 308 siblings of the affected children had malformations similar to those of the affected children. The incidence of these malformations in siblings of affected children is significantly greater than the incidence in the general population.

In recent years malformations of the central nervous system have been studied from many points of view, well summarized by Penrose (*1*). Studies of the familial aggregation of these malformations, however, have been few in number. Polman (*2*), investigated the families of 105 anencephalics and found 13 sibs with anencephalus and 11 sibs with spina bifida or other malformations of the central nervous system. Böök and Rayner (*3*) found no affected sibs in 46 anencephalic sibships. Penrose (*1*) found one anencephalic and two spina bifida cases in 21 anencephalic sibships. This report is concerned with a study of 139 sibships of anencephalic and spina bifida cases.

Table 1. Outcome of pregnancies in patients having two or more infants with malformations of the central nervous system. Abbreviations: *Ab.*, abortion; *An.*, anencephalic; *E*, encephalocele; *H*, hydrocephalic; *M*, meningocele, *Mm*, meningomyelocele; *N*, normal; *P.D.*, premature, died; *S.B.*, spina bifida.

Patient number	Pregnancy number									
	1	2	3	4	5	6	7	8	9	10
1	Ab.	S.B.*	N	An.*	N					
2	N	P.D.	P.D.	An.*	N	Mm*	P.D.	N		
3	P.D.	N	N	An.†	N	N	N	N	An.*	An.‡
4	N	N	N	E*	N	N	An.*			
5	N	N	M*	S.B.*	N					
6	P.D.	P.D.	§*	S.B.*	N					
7	H‖	Ab.	Mm*							
8	S.B., H*	N	M*							
9	An.*	N	M*	N						
10	N	An.*	An.*							

* Ascertaining case. † Born outside study area. ‡ Born outside study period. § Premature twins, one with spina bifida. ‖ Not reported on birth certificate.

The records of birth and stillbirth filed at the New York State Department of Health and records of the three maternity hospitals in Albany, New York, were used as source material.

Sibships were ascertained by the following criteria. A case was defined as an infant born at one of the three maternity facilities in Albany, New York, in the years 1945 to 1960, with anencephalus or spina bifida (spina bifida, meningocele, meningomyelocele, myelocele, rachischisis) reported on the certificate of birth or stillbirth. Two procedures were followed to locate the sibs of each case: the hospital record of the mother of each case was searched and her pregnancy history was recorded, and the New York State Department of Health birth indexes were searched for the births noted on the hospital record and for any other births recorded to the mother. In this way all recorded births to these mothers which occurred in upstate New York were discovered.

Copies of the birth records of the index cases and their sibs were assembled into sibships and analyzed. Sixty-four anencephalic and 75 spina bifida sibships were assembled. Three hundred and eight siblings of index cases were found in these families, counting only one index case per family. Nine of the 139 families had a second child with a central nervous system malformation, and one family had a second and a third child with a central nervous system malformation. Five families had a second child with malformation not of the central nervous system. The ten sibships with two or more malformations of the central nervous system are presented in Table 1. In these families 24 of 53 known pregnancies had a normal outcome. This is in marked contrast to the findings of Labrum and Wood (*4*) who found only 4 of 30 pregnancies normal in ten families having two or more malformed infants.

The incidence of anencephalus and spina bifida in upper New York state over the years of this study was 200 cases per 100,000 live and stillbirths. The population incidence was determined with precisely the same definition of a case as was used in ascertaining cases in the sibships. Applying this rate to the 308 siblings one would expect less than one case (0.6) of malformation in this number of births. Actually, ten cases were observed, a statistically significant excess. Eight of the ten cases in sibs were primarily ascertained. One case was born outside of the study area, and one case was born out of the study period.

These findings support the conclusion that anencephalus and spina bifida have a much higher incidence among siblings of affected children than in the general population. On a geographic basis, the highest malformation rates per birth were overwhelmingly confined to low socioeconomic areas in the oldest sections of Albany. This is consistent with Edwards' (*5*) finding of an increased incidence of malformations of the central nervous system in lower socioeconomic groups.

No aggregation in year or month of birth of the affected cases was noted.

In New York state more than 99.4 percent of births occur in hospitals and are reported to the Department of Health. This obviates bias which could

arise from differences between hospital and nonhospital births. Also, a recent analysis of the reporting of spina bifida at the New York State Department of Health (*6*) reveals at least 85 percent complete reporting of the defect on birth and stillbirth certificates. There is no reason to assume that the reporting of anencephalus is any worse, since this defect is so easily recognized.

It could be argued that a family with two malformed infants would be more likely to be ascertained than a family with one malformation. Granting this possibility, the difference between the population incidence of anencephalus and spina bifida and the incidence in sibs would still be significant.

A bias does exist in that some of these sibships are still incomplete. Sibs born after the index cases are therefore underestimated. Similarly, families which have moved out of the reporting jurisdiction while in the childbearing period will also produce an underestimate of cases. Ten of 308 siblings affected may therefore be a low estimate.

The findings of this study are in close agreement with those of Polman and Penrose. They suggest the importance of familial factors, genetic or environmental, in the causation of anencephalus and spina bifida (*7*).

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Experimental Study of the Developing Mammalian Egg: Removal of the Zona Pellucida

Abstract. The zona pellucida may be removed from all stages of the mouse egg by digestion with pronase. Cumulus and corona cells are also dispersed by the enzyme. No change in membrane disgestibility occurs at fertilization. In tests thus far of two-cell eggs and later stages, development continues. Blastocysts exhibit "hatching" behavior despite absence of the zona. Functions of the zona include maintenance of the normal cleavage pattern and prevention of egg fusion.

The fact that the mammalian egg has thus far served as an experimental subject only to a relatively limited extent, despite its great potential interest, can be ascribed at least in part to persistent technical difficulties. We have successfully overcome a number of these difficulties in the case of the mouse egg; this report describes the procedure for removing the zona pellucida. Among the problems now more accessible to investigation are the mechanisms of sperm penetration and fertilization, the causes of polyspermy, the effects of large molecules or disease entities which the zona might ordinarily exclude (*1*), and the role of the parts of the developing egg in morphogenesis.

The zona pellucida is slightly elastic and can be peeled off with a pair of glass needles, but the process is tedious and, like other kinds of manipulation toward the same end, often results in injury to one or more of the early blastomeres. According to Braden the membrane is composed of neutral or weakly acid mucoprotein (*2*). Though it can easily be dissolved by a variety of treatments, viability has been seriously impaired in all cases in which egg survival has been observed. Effective agents, with some species differences among laboratory mammals, include low *p*H (<5), denaturants, strong oxidizing or reducing compounds, and a number of proteolytic enzymes (trypsin, chymotryspin, ficin) (*2*). Use of a mold protease was also mentioned by Braden in the rat (*2*), but its influence on the egg was not noted. In preliminary tests we verified earlier work on the mouse and added lysozyme, elastase, collagenase, and hyaluronidase to the list of products ineffective in this species. The assembled information does not, however, support any clear conclusions regarding the chemical nature of the membrane.

More recently, we have discovered that the enzyme pronase (*3*) is highly efficient at removing the zona pellucida of the mouse egg at all stages of development. It is, moreover, the only substance to date which apparently does not damage the egg during the brief exposure required (*4*). The enzyme was first isolated in Japan from a strain of *Streptomyces griseus* and was of interest because of its wide substrate specificity (*5*).

Digestion of the mouse egg zona with pronase occurs readily under a wide range of conditions. The following standard procedure has been adopted. A 0.5-percent solution of the enzyme is made up in bicarbonate-free Hanks balanced salt solution. Insoluble mycelial impurities are removed during sterile filtration. The solution remains stable for at least a week under refrigeration. Shortly before use, it is warmed to 37°C under 5 percent CO_2 until the *p*H, which is initially high, is approximately neutral. Rinsing the eggs in protein-free medium before transfer is unnecessary. Further incubation is at room temperature. Though the solution cools, digestion time is very constant at close to 3 minutes. A medium for in vitro cultivation of two-cell or older eggs has been devised (*6*). The eggs are washed four times in this medium, and their development is observed after further incubation.

The so-called zona reaction has often been discussed as a possible means of rendering polyspermy less likely in some species, including the mouse (*7*). According to this hypothesis, a change in the zona pellucida occurs upon fertilization and inhibits penetration by more than one sperm. It was therefore of interest to compare the rates of hydrolysis of the zona in the one-celled egg before and after fertilization. Rates of digestion by pronase in the two stages were, however, identical. The follicle cells of the cumulus oophorus and corona radiata which still surround the uncleaved egg are instantaneously dispersed by pronase, and their intercellular matrix is dissolved. Thus the occurrence of a protein constituent in addition to the hyaluronic acid known to be present is confirmed. Fertilized eggs denuded before cleavage are being tested for normalcy of development by reimplantation into pseudopregnant females.

Two-cell eggs usually show abnormal cleavage patterns in vitro after the zona is removed; this appears to be due to inadequately developed cell adhesiveness, as a result of which the blastomeres form loosely bound flat plaques or random arrangements (*8*). They may, however, round up and form a morula which continues development. Eight-cell eggs deprived of the zona progress in vitro in a com-