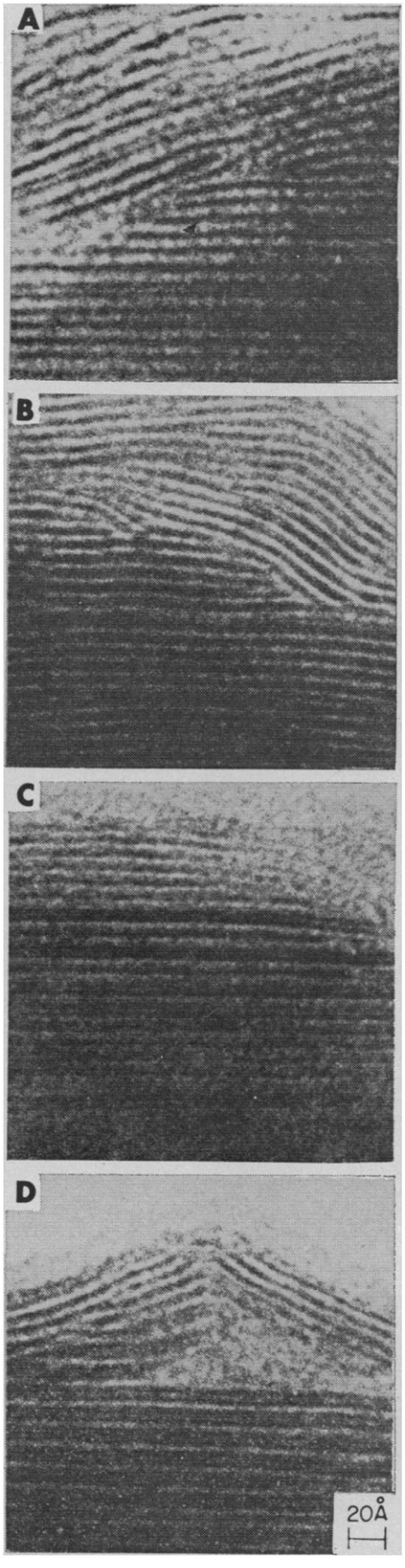

Fig. 2. Dislocations and other crystal lattice imperfections directly resolved by electron microscopy in thin specimens of (A and B) $TaS_2(C_5H_5N)_{1/2}$ showing edge dislocations, associated stacking faults, and first-stage regions alternating with second-stage regions in (C and D) $TaS_2(C_5H_5N)_{1/4}$. (Magnification, × 2,250,000.)

visualization of the structure of the crystalline lattice of layered intercalation compounds down to the atomic level, which can be further extended to the direct study of materials in the superconducting state. Cryo-electron microscopy at liquid-helium temperatures (*6, 10*) may also be used to observe trapped flux patterns by the decoration techniques of Träuble and Essmann (*11*) or through direct visualization of magnetic distribution (*10, 12*) and detection of regular arrays of flux lines in thin films of type II superconductors by electron diffraction (*13*).

Finally, the demonstration presented here of atomically thin metallic layers separated by an organic barrier is also of interest in connection with the general problem of superconducting tunneling (*14*). The multilayered structures of atomic dimensions depicted here are well within the range of tunneling phenomena and might exhibit characteristics related to Josephson tunneling (*15*) in novel ways uniquely derived from the three-dimensional configuration of the layered superconducting intercalation complexes.

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Buffer Combinations for Mammalian Cell Culture

Abstract. *The growth and metabolism of cultured mammalian cells are markedly affected by the* p*H variation in ordinary bicarbonate-buffered media (*p*H 8.0 to 6.9). Those* p*H swings can be reduced and the* p*H of the culture can be stabilized as desired in the range* p*H 6.4 to 8.3 by appropriate combinations of two or three organic buffers, each at 10 to 15 millimolar, in conjunction with phosphate and bicarbonate. The initial alkalinization in sparse cultures is then minimized, and the metabolic acidification in 24 hours is usually less than 0.4* p*H unit except in heavy cultures.*

Relatively minor variations in the *p*H of the medium in the range *p*H 6.8 to 8.2 markedly affect the growth of normal, virus-transformed, and cancer cells (*1, 2*). The optimum *p*H varies with the individual strain, from as low as *p*H 6.9 to 7.1 to as high as *p*H 7.6 to 7.8 (*2*). In $NaHCO_3$-buffered media, an initial loss of CO_2 in sparse cultures raises the *p*H to approximately 8.0. This shift is followed by acidification as a result of cell metabolism, with a *p*H after 24 hours as low as 6.9 in heavy cultures. This prolonged exposure to suboptimal *p*H accentuates the sensitivity of normal diploid cells to a growth inhibitory effect of cellular interaction, which develops at relatively low population densities ["contact" inhibition of growth (*3, 4*)]. This population-dependent inhibition can be promoted by appropriate variations in the *p*H of the medium, and can be at close to optimal levels, with a resulting large increase in the maximum population density achieved by normal cells (*2, 5*). Not only the growth of the cells, but other parameters of cellular metabolism may also be affected by *p*H variation (*6*).

The stabilization of *p*H in cell cultures is of some importance. The com-

bination of 40 m*M* HEPES and 10 m*M* TRICINE used in a previous study (*2*) adequately covers only a portion of the desired *p*H range and has proved toxic for some cell lines. Good *et al.* (*7*) have studied the effects of a group of nonvolatile organic buffers (pK_a 6.5 to 8.3) in biological systems (chloroplasts and mitochondria), and additional buffers in the same range have since become available (Table 1). As is shown here, of the 16 compounds studied, eight proved relatively nontoxic at a concentration of 10 to 20 m*M* for four different cell lines (Table 2). In appropriate combination and in conjunction with phosphate, these permit a reasonably effective stabilization of culture *p*H at any desired level between *p*H 6.4 and 8.3.

The medium used in the initial experiments was a minimal essential medium (*8*) that contained 24 m*M* (0.2 percent) sodium bicarbonate as the major buffering system and was supplemented with nonessential amino acids (0.2 m*M* each) and 5 percent each of calf serum and fetal calf serum. The medium (*8*) that contained 24 m*M* added as an essential ion; higher concentrations of this ion could not be used because of its precipitation as Ca salts, particularly during the initial period of alkalinization in young cultures.

The cells used included two normal human fibroblasts (strains KL and MS2, isolated by E. M. Levine and M. Siniscalco, respectively); two human cancer cells (KB and HeLa); a mouse fibroblast (L-929); and two virus transformants—AGMK-adeno, a monkey kidney cell transformed by adenovirus, and WI 26-VA, a human fibroblast transformed by SV40 (*9*). Methods of cultivation (*4*, *9*) and methods for the determination of cell protein (*10*) and cell number have been described.

Of the buffers evaluated (Table 1), eight were excluded from further consideration on the basis of marked toxicity at concentrations of 20 to 40 m*M*. The remaining eight compounds were not significantly toxic at 20 m*M* for the cell lines studied. The effects of four of these in varying combination on the buffering capacity of a growth medium supplemented with 10 percent serum is shown in Fig. 1. Table 3 lists suggested combinations for specific p*H* ranges. The increased tonicity of the medium caused by the total 30 to 40 m*M* buffer did not of itself inhibit the growth of the cell lines here studied (*11*). A minor toxicity has, however, been observed with some cell strains, evidenced in the decreased growth, for example, of rabbit lens epithelium and a rat liver cell. In such cases, it is suggested that the buffer concentrations indicated in Table 3 be reduced to one-half or one-fourth of the concentrations there indicated. A similar reduction in buffer concentrations is suggested for the initiation of primary cultures from freshly trypsinized tissues or tissue fragments, in which there is no a priori information with respect to toxicity. As indicated in the last column of Table 3, a combination of three of these buffers (BES, HEPES, and HEPPS) may be used to provide a measure of *p*H control in the range *p*H 7.0 to 8.0.

Although phosphate ion is essential for mammalian cells (*11*), and although high concentrations (10 m*M*) can be used for suspension cultures in Ca-free media (*8*), the concentration in media for monolayer cultures is limited by the precipitation of the Ca salt during the initial alkalinization of

Table 1. Buffers tested for toxicity and buffering activity in mammalian cell cultures.

Buffer name	Chemical name	pK_a	Refs.
MES	2-(*N*-morpholino)ethanesulfonic acid	6.15	(*4*)
BIS-TRIS	[bis-(2-hydroxyethyl)imino]-tris-[(hydroxymethyl)methane]	6.46	(*)
ADA	*N*-(2-acetamidol)iminodiacetic acid	6.60	(*4*)
Bis-tris-propane	1,3-bis[tris(hydroxymethyl)amino]propane	6.8	(†)
PIPES	Piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)	6.80	(*4*)
ACES	*N*-(2-acetamidol)-2-aminoethanesulfonic acid	6.90	(*4*)
Imidazole	Imidazole	7.0	
BES	*N*,*N*′-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid	7.15	(*4*)
MOPS	Morpholinopropanesulfonic acid	7.20	(*)
TES	*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid	7.50	(*4*)
HEPES	*N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid	7.55	(*4*)
HEPPS	*N*-2-Hydroxyethylpiperazinepropane sulfonic acid	8.00	(*)
TRICINE	*N*-tris(hydroxymethyl)methylglycine	8.15	(*4*)
Glycinamide	Glycinamide	8.2	
TRIS	tris(hydroxymethyl)aminomethane	8.3	(*4*)
BICINE	*N*,*N*′-bis(2-hydroxyethyl)glycine	8.35	(*4*)

* Reference material is available from Schwarz-Mann, Nutritional Biochemical, or Calbiochem, whichever is the supplier. † Supplied by Dr. Norman Good.

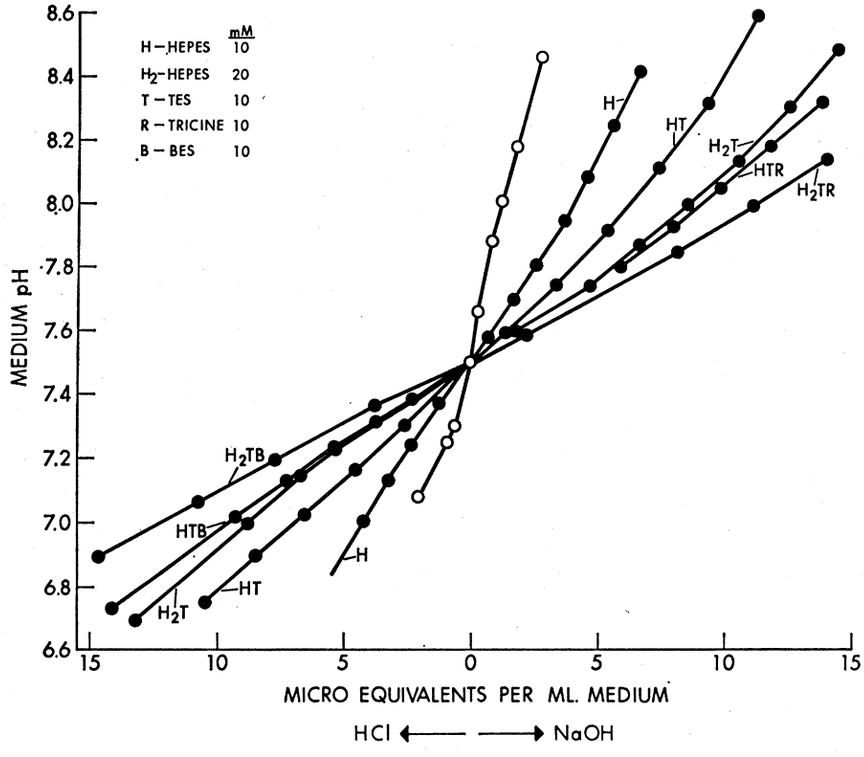


Fig. 1. Titration curves of complete growth medium buffered with varying combinations of HEPES, TES, TRICINE and BES, in addition to 24 m*M* $NaHCO_3$. The open circles indicate the titration curve of medium containing $NaHCO_3$ alone. HTR was titrated only with alkali, and HTB only with acid.

Table 2. Buffers not significantly toxic for mammalian cells at 20 m*M* concentration. Growth is expressed as percentage of growth in control medium containing no organic buffer, but adjusted at time of medium change to *p*H 7.4.

Buffer	pK_a	Growth after 6 to 7 days (%)				
		KL	HeLa	WI26-VA	AGMK-adeno	929
BIS-TRIS	6.46	100	96	100	88	96*
PIPES	6.8	98*	75*	89*	78*	100
BES	7.15	111*	94		91,56*	104
MOPS	7.2	96	75*	88	88*	100
TES	7.5	100	99		86*	101
HEPES	7.55	100	95	108	90*	100
HEPPS	8.0	100	92*	95	96*	88
TRICINE	8.15	100*	100	100	92	116

* Minor toxicity at 40 m*M* level (growth 60 to 80 percent of control). The apparent failure of the organic buffers to promote growth reflects the fact that these are short-term results, preceding "contact" inhibition.

Table 3. Buffer concentrations (m*M*) recommended for media at indicated *p*H. Concentrations of organic buffers should be reduced if toxicity is noted for specific cell line, and reduced also for primary cultures, direct from tissues. Buffers added are in addition to the $NaHCO_3$ of the medium.

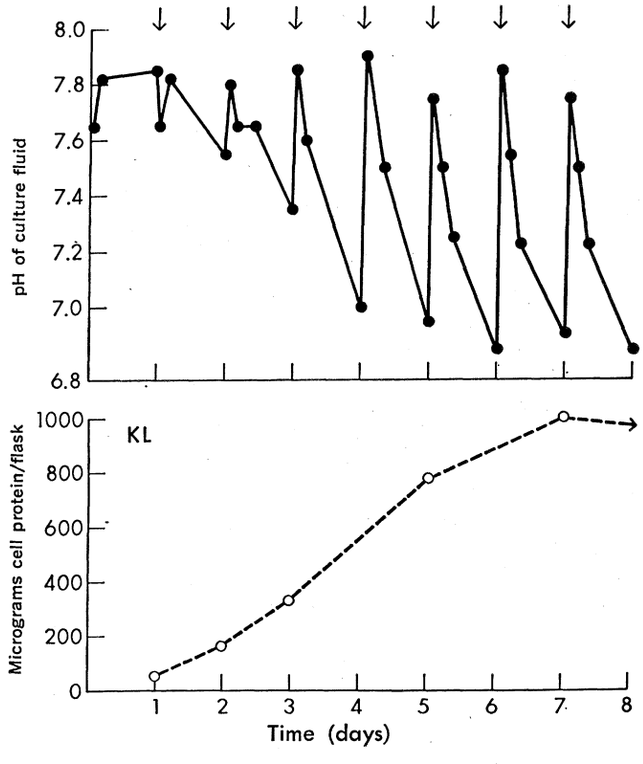
Buffers	pK_a	Buffer concentrations (m*M*) at *p*H:							For general use*
		6.5	6.8	7.1	7.4	7.7	8.0	8.3	
BIS-TRIS	6.46	10†							
PIPES	6.8	10	10	10					
BES‡	7.15	10	15	10	10				10
TES	7.5				10	15	10		
HEPES	7.55			10	15	15	15		15
HEPPS (EPPS)	8.0						10	15	10
TRICINE	8.15							15	
NaH_2PO_4		10	10	5	2				

* Although this buffer combination is moderately effective over the *p*H range 7.0 to 8.0 the *p*H fluctuations will be somewhat greater than with the combinations suggested for a specific *p*H range. † Conveniently added as 1 percent of 1*M* stock solution. Some of these buffers are strongly acidic or basic, and medium must be adjusted to desired *p*H with NaOH or HCl. ‡ May be substituted by MOPS, with a pK_a of 7.2.

the culture (Fig. 2). Between *p*H 6.3 and 7.2, however, this does not pose a problem, and NaH_2PO_4 may be added to supplement the organic buffers (Table 3).

*p*H *Variation in Buffered Media.* In Fig. 2 are shown the *p*H changes in the course of a single culture passage in stoppered flasks when the usual $NaHCO_3$-buffered media are used. Within 1 hour after the inoculation of a trypsinized cell suspension into a stoppered flask at a final concentration of 1.5×10^3 cells per square centimeter, the *p*H had risen from 7.4 to 7.9 because of the loss of CO_2, and remained essentially unchanged over the next 24 hours. After a medium change, the *p*H slowly decreased to 7.55 over the next 24 hours; and with daily feedings thereafter, the rate and degree of metabolic acidification progressively increased, reflecting cellular multiplication. The duration of the initial period of alkalinization and the rate of the following acidification varied from cell to cell and, understandably, varied also with the size of the inoculum.

Figure 3 illustrates for two cell lines the degree to which these *p*H fluctuations were controlled by the buffer combinations indicated in the figure legend. The intial alkaline shift in sparse cultures was minimized, and the



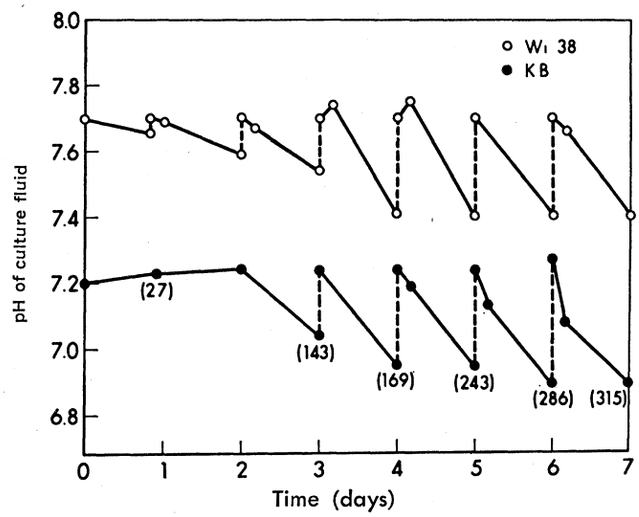


Fig. 2 (left). Variations in *p*H in a human fibroblast (KL) culture (growth medium buffered with 24 m*M* $NaHCO_3$). Fig. 3 (right). Variations in *p*H in medium containing nonvolatile buffers. At *p*H 7.7, the medium was buffered with 10 m*M* HEPES, 20 m*M* HEPES, and 10 m*M* TES. At *p*H 7.2, the medium was buffered with 10 m*M* HEPES, 10 m*M* TES, and 20 m*M* BES. The open circles show the control of *p*H in a normal human fibroblast (WI 38) culture. The half-shaded circles indicate the control of *p*H fluctuations in a human cancer (KB) culture. The vertical dotted lines indicate replacement of culture fluid with fresh medium. Numbers in parentheses in lower curve indicate the number of micrograms of cell protein per square centimeter of culture surface.

metabolic acidification in a 24-hour period ranged from 0.1 to 0.4 *p*H unit, depending on the specific cell and the population density. The development of additional buffers with a pK_a of 7.5 to 8.0 would further limit that acid shift. A slow alkaline shift in media at *p*H 6.2 to 6.8, due to loss of CO_2, can be reduced by decreasing the concentration of bicarbonate in that range, from 24 m*M* to, for example, 5 m*M*.

A special problem in *p*H control is introduced when cells must be grown in containers open to the air (such as petri dishes) in a CO_2 incubator. With ordinary bicarbonate-buffered media, the final *p*H is determined by the concentrations of $NaHCO_3$ in the medium and of CO_2 in the atmosphere, subject only to a time lag in CO_2 diffusion. The *p*H cannot be flexibly adjusted with nonvolatile buffers, since the final *p*H will then be intermediate between the initial *p*H of the medium and that imposed by the CO_2-$NaHCO_3$ system; nor can the $NaHCO_3$ be eliminated, since added bicarbonate is an essential metabolite for many cell lines (*12*). However, the two buffer systems can be made mutually supportive by adjusting the concentration of $NaHCO_3$ to that level which in conjunction with a fixed atmospheric CO_2 concentration gives approximately the desired *p*H (*1, 13*). The combination of buffers shown in Table 2 can then be added in order to enhance the buffering capacity of the medium.

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Juvenile Hormone Induces Vitellogenin Synthesis in the Monarch Butterfly

Abstract. *The vitellogenin (yolk protein) of the monarch butterfly has been identified by electrophoretic and immunochemical techniques. In freshly emerged female adults ligature of the neck prevents appearance of this protein in the hemolymph; it prevents yolk deposition and egg maturation as well. These processes are restored by injection of juvenile hormone; the restoration involves induction of vitellogenin synthesis, as shown by incorporation of* [^3H]*leucine.*

Yolk formation in insects entails the selective uptake of vitellogenins ("female proteins") from the blood into the oocytes (*1*). Vitellogenin is synthesized by the fat-body cells, and in insects of several orders other than Lepidoptera (moths and butterflies) this synthesis is induced by juvenile hormone secreted by the corpora allata (*2*). Although earlier studies on Lepidoptera (chiefly with silkmoths) showed that allatectomy failed to prevent yolk formation (*3*), recent reports on other members of this order clearly demonstrate dependence on the corpora allata (*4*). Until now, however, nothing has been reported on the mode of action of the juvenile hormone in controlling egg maturation in any lepidopteran. To investigate this, we selected the monarch butterfly *(Danaus plexippus)* because the occurrence of both a reproductive phase and a sexually dormant migratory phase in its long-lived adults suggested the existence of hormonal regulation.

Initially, we looked for the presence of the vitellogenin in this species. Larvae were reared in the laboratory on either milkweed leaves or an artificial diet under 16 hours of illumination per day at 25°C; adult butterflies were fed twice daily on diluted honey (*5*). Antiserum against vitellogenin was obtained from a rabbit immunized with the proteins of mature eggs (*6*). Hemolymph was collected from animals at various developmental stages and analyzed by immunoelectrophoresis (Fig. 1). The only sample which produced a major precipitin line identical to that from yolk and representing vitellogenin was the blood of the female adult in which vitellogenesis was in progress. Neither males at any stage of development nor pupal or newly emerged adult females contained this protein. These results were further confirmed by results of the immunodiffusion tests of Ouchterlony and Oudin in which antiserum absorbed with male blood was used (*6*), and by disc electrophoresis on acrylamide gel.

To test hormonal control over yolk formation, we fed seven newly emerged adult females once and then ligated four of them at the neck, thus cutting off the brain and associated neuroendocrine glands from the rest of the body. The remaining three served as controls. All were then maintained for 5 days by a daily injection of glucose solution into the hemocoel. Hemolymph and ovaries were then examined. The results (Table 1, experiment 1) were clear-cut, and indicated that an endocrine center in the head of the butterfly is required for the appearance of vitellogenin, and therefore for the deposition of yolk and egg maturation.

A second experiment was designed to test whether control over yolk production was a function of juvenile hormone, a product of the corpora allata,

Table 1. Effects of ligation and treatment with juvenile hormone on vitellogenin production and egg development in the monarch butterfly. Female butterflies, within 12 or 24 hours after emergence, were fed on 30 percent honey and immediately ligated at the neck (except for the intact controls of experiment 1). In experiment 2, juvenile hormone (JH) or oil was injected. The animals were then kept at 25°C and 60 percent relative humidity, and maintained by daily intraabdominal injection of 50 to 80 μl of 2 or 5 percent glucose solution. Finally, hemolymph samples were collected, and the abdomens were dissected and examined. Vitellogenin in the hemolymph was determined by the Oudin immunodiffusion test and other techniques as described in the text (+, present; −, absent). Vitellogenesis in the oocytes was determined by visual examination (+, oocyte growth and presence of abundant opaque yolk; −, no more than traces of opaque material). Newly emerged butterflies also had only traces of opaque material. The juvenile hormone used in experiment 2 was *dl*-JH (*dl*-methyl *t,t,c*10-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate), kindly given by Dr. H. Röller. It was dissolved in olive oil (1 μg/μl) and 4 μl were injected; controls received 4 μl of olive oil.

Treatment	Animals (No.)		Vitellogenin	Vitellogenesis	Mature eggs
		Experiment 1			
Control	3	5	+	+	+
Ligated	4	5	−	−	−
		Experiment 2			
Olive-oil	3	2.5	−	−	−
JH	2	2.5	+	+	−
JH	1	4.5	+	+	+