jected on the 9th day of incubation. Both groups were harvested and assayed on the 10th day. It was found that transamidinase was repressed to 43% of water-injected control values 1 day after creatine injection, and to 11% of control values 2 days after creatine administration.

Discussion. It is particularly significant for theories of differentiation that repression can be shown experimentally to occur during embryonic development. Heretofore it has been difficult to modulate enzyme levels prior to birth(4), although some success with hormonal induction has been reported(5). Monod and Jacob(6), using hypothetical circuits composed of known control elements, have described how interlocking, individually reversible, repressions might combine to give essentially irreversible differentiation. In their circuits, brief exposure to a single compound could determine subsequent enzyme patterns (*cf.* 7). During development, not only might individual gene function be modulated by cross repression or end-product repression, but whole groups of genes may be turned off or on at the proper time(8) by action of hormones, histones(9), or cross-reacting regulator systems(6).

Summary. Modification of the physical state of injected creatine has permitted a quantitative study of repression of arginine-glycine transamidinase by creatine, and its precursors, in liver of the developing chick embryo. Half-maximal repression was obtained by 65 μmoles/egg of creatine and 45 μmoles of guanidinoacetate. Complete repression occurred with 260 and 120 μmoles, respectively. Betaine and 4-aminobutyrate decrease the degree of repression attained. Arginine, and its precursor, citrulline, repress 50% at 290 μmoles/egg; when glycine is injected with either of these compounds, repression is increased to 80%. Ornithine, which cannot be converted to citrulline in the chick, does not repress. Laying hens fed creatine and guanidinoacetate have embryos with normal transamidinase levels. For the first time, essentially complete enzyme repression has been attained throughout embryonic development. However, derepression occurs during the 6 days following hatching, when chicks are fed a complete diet. In absence of food, or in presence of dietary creatine, this increase does not occur. The possible significance for theories of differentiation of the demonstration of enzyme repression during embryonic development is discussed.

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Received October 22, 1962. P.S.E.B.M., 1963, v112.

Effect of Cold Passage on Reproductive Capacity at Various Temperatures of Several Type 3 Poliovirus Strains.* (28007)

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The reproductive capacity of poliovirus strains varies depending upon the temperature of incubation during the replication cycle(1, 2). Strains vary, one from another, with regard to the temperature at which their repro-

* This investigation was supported (in part) by USPHS Grant from Nat. Inst. of Allergy & Infect. Dis.

ductive capacity is greatest. There is also variation in the range of temperatures at which various strains replicate to an appreciable extent. For example, there are strains that replicate as well at 40°C as at 37°C, while other strains have a much lower reproductive capacity at 40°C than at 37°C(2,3,4).

It has been possible to alter a strain's reproductive capacity at a particular temperature by serial passage in tissue cultures that were maintained at a different temperature. Thus, the passage of a strain at 40°C or higher causes a marked increase in the growth potential of that strain at 40°C(1). In similar fashion, passage in the cold yields strains that replicate as well or better at low incubation temperatures as at 37°C(5,6).

The strains that have been chosen previously for serial passage in the cold were usually those that already showed little growth at 40°C; following passage at 23°C, their reproductive capacity at 40°C was reduced still further.

Plotkin *et al.*(6) found that the genetic marker characteristics of a type 3 poliovirus strain which had undergone passage at 23°C were more stable during human intestinal passage. This technic was also used to increase the stability and reduce the pathogenicity of a type 2 strain(7).

In the study of the effects of passage in the cold on the reproductive capacity of a strain, 3 parameters are important: 1) the characteristics of the strain prior to passage, 2) selection effects that occur during passage, and 3) characteristics of the strain after passage. The investigations reported here involved examination of the growth potential of several wild strains of poliovirus at 23°C, 37°C and 40°C. The study included determination of the reproductive capacity of plaque pools that had been obtained from the test strains. The effects of serial passage at 23°C upon the reproductive capacity of the test strains and of their plaque pools were also determined. Several of the pools obtained during each passage series at 23°C were examined for their reproductive capacity at different temperatures. It was hoped that a more complete understanding of the effects of cold passage could be obtained by combining the study of reproductive capacity of plaqued particles with examination of the pools obtained from a strain during the series of passages at 23°C.

Materials and methods. Viruses. The viruses used in these experiments were: W-Fox (8), an attenuated type 3 strain; Habel-24, a virulent type 3 strain; and 2 wild strains (L-38 and L-97) that were isolated from cases of poliomyelitis in Louisiana by Gelfand(9).

Cell cultures. Monkey kidney tube cultures were prepared by seeding each tube with 3×10^5 cells suspended in 0.5 ml Hanks' BSS containing 0.5% lactalbumin hydrolysate and 2.0% calf serum in Hanks' BSS. After 4 days incubation, the medium was changed to Earle's BSS containing 0.5% lactalbumin hydrolysate and 2.0% calf serum. Before inoculation of the tubes with virus, this medium was replaced with 0.9 ml of Eagle's medium in Earle's salts for maintenance. Streptomycin (100 μg/ml) and penicillin (100 units/ml) were included in all media used for cell growth or maintenance in these experiments.

Titration technics. Titrations in monkey kidney tube cultures were done by adding 0.1 ml of each of the desired dilutions to 3 tubes. Dilutions were done in Hanks' BSS. Virus titers were determined by the 50% end-point technic(10).

Plaque isolation and passage. Plaques were picked, dispensed into 1 ml of Eagle's in Earle's medium and stored at −20°C. Each plaque pool was passed in a monkey kidney tube culture by inoculation of 0.1 ml of plaque suspension per tube. When the cytopathic effect was complete, the samples were harvested and stored at −20°C.

Maintenance of temperature. In studies of the reproduction capacity of polioviruses, the maintenance of temperature is of prime importance. For the cultures kept at 40°C, waterbaths were equipped with a heating and agitating apparatus (Mergotherm, H. Struers Chemiske Laboratorium), which maintained temperature within ±0.02°C of the desired temperature throughout the bath. The rack containing the infected culture tubes was submerged in the water where it remained during the entire incubation period.

A temperature of 23°C was obtained by placing an incubator in a room in which the ambient temperature was 4°C. This incuba-

TABLE I. Reproductive Capacity of Several Type 3 Poliovirus Strains at 23°C, 37°C and 40°C.

Virus strain	Description of strain	Titer in $\log_{10}$ units/ml at 23°C	37°C	40°C
H-24	Virulent†	<.7	8.0	8.2
W-Fox	Attenuated†	<.7	7.5	4.0
W-Fox 22 TCP* at 23°C	Passage of W-Fox	5.5	5.5	<.7
L-38	Wild	2.0	7.0	7.1
L-97	"	3.2	8.0	8.0

* TCP = tissue culture passage.
† Classification of virulent or attenuated was determined by inoculation into monkeys.

tor maintained the temperature within ± 0.3°C of 23°C.

Results. Several type 3 strains were tested for their growth at 23°C, 37°C and 40°C. The strains were titrated in monkey kidney tubes and 3 tubes from each dilution were then placed at each of the 3 temperatures. The results (Table I) indicate marked variation in the reproductive capacity of the 5 strains. The Habel-24 strain titered as high at 40°C as at 37°C, while the W-Fox strain replicated poorly at 40°C compared to 37°C. Neither strain had any capacity to replicate at 23°C. A third sample tested was the pool obtained after the 22nd passage at 23°C of virus isolated from a human that had been fed W-Fox strain. The procedure used for the serial passage in the cold was as follows: a pool of virus was prepared from the stool suspension by passage at 37°C in monkey kidney tissue culture tubes. This virus pool was then inoculated into culture tubes (0.1 ml/tube) that were maintained at 23°C throughout the incubation period. During the first 2 passages there was no cytopathic effect after 10 days of incubation. On the third passage, a cytopathic effect appeared after 3 days. When the cytopathic effect was complete, the pool was harvested, frozen at –20°C and subsequently passed at 23°C by inoculation of 0.1 ml of undiluted material in each monkey kidney culture tube. The virus pool produced after the 22nd passage at 23°C was titrated at 23°C, 37°C, and 40°C. It was found that this pool replicated as well at 23°C as at 37°C and had no capacity for growth at 40°C.

Two wild strains, L-38 and L-97, were titrated at the 3 temperatures. These 2 strains grew as well at 40°C as at 37°C. The titers of the 2 strains at 23°C were quite low compared to their titers at 37°C and 40°C. Each strain, however, caused cytopathic effects at a higher dilution than was noted with the W-Fox or Habel-24 strains (Table I). The L-97 and L-38 strains were then passed in monkey kidney culture tubes that were maintained at 23°C.

The L-38 strain was passed 14 times at 23°C by the same technic outlined in an above section. The original pool and the 14th passage pool were titrated in monkey kidney tube cultures at 23°C, 37°C, and 40°C. The results are shown in Table II. After passages at 23°C, the pool had lost most, but not all, of its capacity to replicate at 40°C.

The original pool and the 14th passage pool were titrated at 37°C on monkey kidney monolayers by the plaque technic, and plaques were picked at random. The plaque pools were then tested for growth at 23°C, 37°C,

TABLE II. Effect of Passage at 23°C on L-38 Virus Replication at 23°C, 37°C and 40°C.

Virus inoculum	Passage history	Titer in $\log_{10}$ units at 23°C	37°C	40°C	Proportion of plaque pools that replicate at 23°C	37°C	40°C
L-38 pool	1 TCP*— 37°C	2.0	7.0	7.1	1/19†	19/19	19/19
Idem	14 TCP — 23°C	7.0	5.3	3.0	18/19	18/18	9/18‡
L-38 PL-16	1 TCP — 37°C	+§	4.2	4.7			
Idem	3 TCP — 23°C	+§	4.7	4.0			
"	4 TCP — 23°C	4.5	5.7	0			
"	5 TCP — 23°C	6.2	4.6	0			
"	7 TCP — 23°C	6.0	2.7	0			
"	10 TCP — 23°C	7.1	1.5	0			

* TCP = Tissue culture passage.
† After 1 blind passage at 23°C, 1 of 19 plaques grew at 23°C.
‡ After 1 blind passage at 40°C, 9 of 18 plaques grew at 40°C.
§ Showed CPE only in the cultures inoculated with undiluted material.

TABLE III. Effect of Passage at 23°C on L-97 Virus Replication at 23°C, 37°C and 40°C.

Virus inoculum	Passage history	Titer in $\log_{10}$ units at 23°C	37°C	40°C	Proportion of plaque pools that replicate at 23°C	37°C	40°C
L-97 pool	2 TCP*— 37°C	—	8.0	8.0	18/19	19/19	19/19
Idem	16 TCP — 23°C	4.5	4.2	0	20/20	18/20	14/20†
L-97 PL-29	1 TCP — 37°C	6.0	7.3	7.3	20/20	20/20	20/20
Idem	8 TCP — 23°C	7.0	6.4	6.0			
"	16 TCP — 23°C	4.5	5.2	1.7			

* TCP = Tissue culture passage.
† After 1 blind passage at 40°C, 14 of the 20 plaques grew at 40°C.

and 40°C. All of the plaque pools obtained from the original L-38 pool grew at 37°C and 40°C, but none showed growth at 23°C. The tissue culture fluid in the 23°C tubes (first tissue culture –23°C) was passed in a second series of tissue culture tubes by the inoculation of 0.1 ml of undiluted material. The second or blind passage pools showed 1 positive—the first tissue culture pool from plaque 16. This pool was then passed at 23°C for 8 additional passages. Several passages were titrated at 23°C, 37°C, and 40°C (Table II). From the third to the fourth passage there was a marked shift in growth potential. The fourth passage had completely lost the capacity for growth at 40°C. Further passages increased the growth potential of the pools at 23°C and decreased their growth potential at 37°C. The plaque pools obtained from the 14th cold passage of the L-38 pool replicated at 23°C and 37°C but failed to replicate at 40°C on initial passage (Table II). After a further, blind, passage at 40°C there was a cytopathic effect obtained from 9 of 18 pools.

Experiments similar to those just described were done with the L-97 strains. This strain was passed 16 times at 23°C. The original pool and the 16th cold-passage pool were titrated at 23°C, 37°C, and 40°C (Table III). The pool obtained after 16 passages at 23°C had lost all capacity for growth at 40°C.

Plaques were produced at 37°C from the unpassed L-97 pool. Eighteen of the 19 plaques tested could grow at 23°C, 37°C, and 40°C. One of these plaque pools, from plaque 29, had titers in $\log_{10}$ units of 4.5, 5.7, and 6.2 at 23°C, 37°C, and 40°C, respectively. Twenty plaques were picked from the titration of plaque 29 at 37°C. These plaque pools were then tested for growth at 23°C, 37°C, and 40°C. All 20 pools showed growth at each of the 3 temperatures (Table III). Plaque pool 29 was passed 16 times at 23°C. The results (Table III) indicate that the passage pool obtained from plaque 29 had not lost very much of its capacity for growth at 40°C even after 8 passages at 23°C. Further passages at 23°C reduced the titer at 40°C but did not eliminate growth at this temperature. In contrast, comparable passages of the unplaqued L-97 pool had eliminated the pool's growth potential at 40°C (see above).

Discussion. The passage of strains at low temperatures of incubation leads to a marked reduction in capacity of the strains to replicate at higher incubation temperatures. The relationship to selective processes is obvious in the case of one of these strains, the L-38 strain. Most of the particles within this strain could not replicate at 23°C; plaque 16, however, exhibited the capacity to replicate at this temperature after one blind passage (Table II). After further passage, this plaque quickly adapted to 23°C. It is easy to understand how particles with this characteristic could become predominant in a population like L-38. Whether these particles appeared because of selection or by mutation during the first passages of the L-38 pool at 23°C is not certain. Most of the particles in the L-97 strain had the interesting ability to replicate at all 3 temperatures of incubation. The process which led to the loss of most of this strain's capacity to replicate at 40°C is less clear than in the case of L-38. However, it is easy to envisage the existence of particles with higher growth potential at 23°C (and lower at 40°C) than infectious units that grow equally well at all 3 temperatures. These particles would then become predominant in

the population. Certainly, this explanation is substantiated by the difference noted in the response of the L-97 pool and plaque 29 pool with regard to their growth potential at 40°C following cold passage. The difference was probably related to the fact that the entire L-97 pool contains particles that have good growth potential at 23°C and little or none at 40°C. In contrast, there were no particles of this type in the unpassed material from genetically homogeneous plaque 29.

The importance of passage in the cold is emphasized by the development of strains with more stable genetic characteristics and with less neuropathogenicity following passage at 23°C(6,7). The relationship between cold passage and stability is not clearly understood. However, reduction in the number of particles with growth potential at 40°C is probably an important factor.

Summary. The reproductive capacity of several type 3 poliovirus strains was studied at 23°C, 37°C, and 40°C. There was marked variation in the capacity of the strains to replicate at different temperatures. Two wild strains, L-97 and L-38, were serially passed at 23°C. After 14-16 passages at 23°C, each strain lost its growth potential at 40°C while its growth potential at 23°C increased. A plaque pool obtained from the L-97 strain had the interesting ability to replicate well at all 3 temperatures. This plaque pool did not entirely lose its growth potential at 40°C even after 16 passages at 23°C. A plaque obtained from L-38 was passed at 23°C. There was a marked alteration in reproductive capacity at various temperatures of this particular plaque pool during a single passage, the third, at 23°C.

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Received October 22, 1962. P.S.E.B.M., 1963, v112.

Modification of an Attenuated Type 2 Polio Vaccine Following Passage at 23°C.* (28008)

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The passage of poliovirus strains in tissue culture maintained at low temperatures of incubation has been shown to have a marked effect upon the genetic characteristics of a virus population(1,2). The establishment of a genetically stable type 3 poliovirus strain (WM-III) following serial passages of an unstable strain at low temperatures has focused attention upon the possibility of applying the cold-passage technic to other strains that appeared to have potential as live attenuated poliovirus vaccines. Experiments in this area have value in the possible development of new strains or modification of existing strains. In addition, the theoretical aspects of the study of cold-passage would be aided by establishing the frequency of occurrence of both the stabilizing and the attenuating effects previously observed.

* This work was supported (in part) by a USPHS research grant from Nat. Inst. of Allergy & Infect. Dis.