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Somatomedin-A bioactivity in rabbit serum after hypophysectomy

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Summary. Hall's bioassay was used to determine the somatomedin (Sm) activity pattern after hypophysectomy in young (40 days) and adult (180 days) rabbits. In the young animals, Sm activity decreased a little, but the serum remained slightly active. The serum of the older rabbits progressively lost its sulphation activity and inhibited SO_4 uptake after 2 weeks following hypophysectomy. The inhibitory activity could not be destroyed by heating. Concomitantly, the same serum continued to stimulate thymidine uptake. From the present results on bioactivity, it appeared that Sm generation was only partially pituitary-dependent, that the pituitary might control inhibitor synthesis, and that the SO_4 and thymidine factors were separate entities, each having its own inhibitor(s).

Introduction.

The initial work of Salmon and Daughaday (1957) on the sulphation factor (SF) demonstrated that hypophysectomy in rats caused a marked drop in the ability of the serum to stimulate cartilage sulphate uptake *in vitro*. Hypophysectomy in humans decreases somatomedin (Sm) activity to 30 to 40 p. 100 of its normal value (Almqvist, Ikkos and Luft, 1961 ; Hall, 1970 ; Phillips, Herington and Daughaday, 1974 ; Herington, Phillips and Daughaday, 1976 ; Takano *et al.*, 1977). In a previous study, Vezinhet (1968) showed that hypophysectomy, carried out before the age of 100 days, did not stop ponderal growth in rabbits ; after that age, it induced arrest of ponderal gain almost immediately. At 40 days rabbit growth rate is maximum (Cantier *et al.*, 1969 ; Beaton, 1976), while at 180 days (the post-puberal period), it has slackened. The aim of the present work is to show that the changes in rabbit SM bioactivity, induced by hypophysectomy, depend on age at the time of the operation.

SM-A was originally characterized by its action on sulphate uptake in embryonic chick cartilage (Uthne, 1973). Later, both Sm-A_1 and A_2 were chemically identified and their amino acid composition defined (Fryklund, Uthne and Sievertsson, 1974). Moreover, not only Sm-A, but also the related peptides, Sm-C, IGF-1, IGF-2 and MSA, were shown to be active in the bioassay (Froesch *et al.*, 1976 ; Gibson *et al.*, 1977 ; Zapf and Froesch, 1977). Up to now, no assay method has been strictly specific for

Sm-A, and the results obtained with bioassays are thus the algebraic sum of Sm-A, Sm-C, IGF, MSA and the inhibitor activities. Therefore, the term, Sm-A, in this paper will refer to this bioactivity measured by the chick embryo cartilage assay.

Material and methods.

Animals — One group of 25 New Zealand male rabbits was operated when 40 days old, and a second group when the 10 males were 180 days old. Only those animals from which blood was taken at least once after hypophysectomy were used. Blood was sampled regularly every week after hypophysectomy until all the rabbits were dead ; some of them were sampled 4 days after surgery.

Hypophysectomy. — The rabbits were hypophysectomized using the parapharyngeal approach and the transphenoidal way, according to the technique of Jacobsohn and Westman (1940) adapted by Vezinhet (1976). The effectiveness of hypophysectomy was monitored during surgery by the appearance of cephalorachidian fluid in the sella, indicating a perfect deconnection of the pituitary stem. Furthermore, the sella of the slaughtered animals was examined under a binocular lens to confirm that no pituitary remnant remained. Post-operative medication was limited to a single hydrocortisone injection (2.5 mg/kg) and a 10 ml glucose (20 p. 100) injection the day of the operation ; the glucose injection was repeated the next day. Hypox rabbits fed *ad libitum* were shown to have a constantly subnormal level of glycemia (Vezinhet Charrier and Dauzier, 1972).

Serum. — The blood was obtained by cardipuncture, allowed to clot at room temperature for about 10 min, and then centrifuged. All samples were stored at — 25 °C until assayed.

Bioassay. — Using Hall's technique (1970), Sm-A bioactivity was estimated by radioactive sulphate uptake into chick embryo pelvis cartilage, with a post-incubation period for reasons explained elsewhere (Charrier, 1978). This type of incubation system supppresses the effects of inorganic sulphate level. Double labelling with $^{35}SO_4$ and tritiated thymidine was used with two sera. The tritiated thymidine was purchased as labelled methyl thymidine from the Commissariat à l'Energie atomique, Gif-sur-Yvette, France, and the $^{35}SO_4$ as carrier-free $Na_2{}^{35}SO_4$ from the Radiochemical Centre, Amersham. The interassay precision, assessed in 10 successive assays for two sera, resulted in the following mean bioactivities : 0.97 ± 0.06 and 0.92 ± 0.08 ($\bar{x} \pm$ sm) with variation coefficients of 18 and 25 p. 100, respectively.

Heating. — Hypophysectomy induces in rat serum an inhibitor of thymidine (Daughaday and Reeder, 1966) or sulphate (Salmon, 1973) uptake in rat costal cartilage. This inhibitor can be destroyed by trypsin (Salmon, 1973) or by heating (Salmon, 1972), and thus when it was found in hypox rabbit serum, we tried to suppress it by heating.

The sera were diluted 50/50 (v/v) with Hall's medium and the pH lowered to 5.5 with HCl. They were then put into stoppered flasks in a cool water-bath, brought

to 100 °C and left boiling for 15 min. The samples were centrifuged after cooling, the proteic clot discarded and the supernatant obtained brought to initial volume by the addition of distilled water. The pH was again raised to 7.45 with NaOH.

Statistical analysis. — Whenever possible, the relative potency of the serum was determined by covariance analysis of a parallel-line assay (Finney, 1964), and its precision estimated by Gaddum's λ index. The standard was a pool of sera from 120-day old rabbits. For the reasons explained below in the results, most serum activity was expressed as a percentage of the controls. As in humans, rabbit Sm bioactivity showed great individual variability. The mean value of twenty-two 40-day old rabbits was 0.50 U/ml (range 0.10-1.02) and of 180-day old rabbits 0.98 U/ml (range 0.44-1.45) (Charrier, 1978).

Results.

Figure 1 shows survival after hypophysectomy in the two groups of animals. In both cases, maximum survival time reached 48 days.

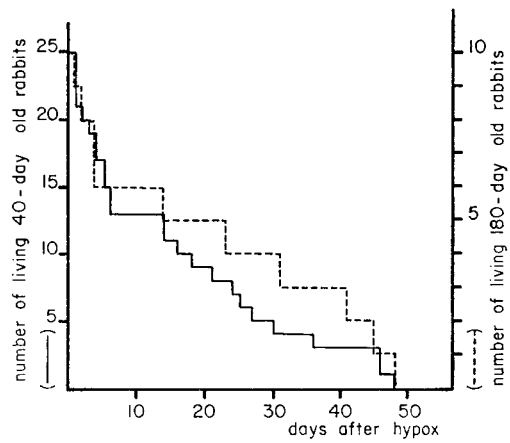


FIG. 1. — *Survival after hypophysectomy of* 40 (——) *and* 180 (------)*-day old rabbits.*

Hypophysectomy led to a progressive drop in serum Sm-A bioactivity with time, as evidenced by decreasing $^{35}SO_4$ incorporation. Figure 2 shows the typical uptake pattern of an adult rabbit : (i) the slope values of the lines decrease with the time-course after hypophysectomy, reach zero level, then become negative ; (ii) the uptake levels slow down with the time-course after hypophysectomy ; (iii) 3 weeks after hypophysectomy, uptake in the serum-stimulated pelvis is below the control baseline, indicating true serum inhibitory activity.

Serum Sm-A bioactivity, determined by the usual 4-point assay, requires that the tested lines be statistically parallel. No estimate can be made if they are not. When the lines were not parallel, as in figure 2, the potency ratio could not be estimated, the results were unclear and many of the data given by the uptake pattern were

masked. To obviate this inconvenience and to acquire a more representative picture, the incorporation obtained with a 20 p. 100 serum concentration was expressed as a percentage of the controls in buffer alone. With this method, the activities could not be expressed as U/ml, but the effect of hypophysectomy on rabbit serum stimulatory capacity was clear, which was the main purpose of this study. The results were therefore presented that way.

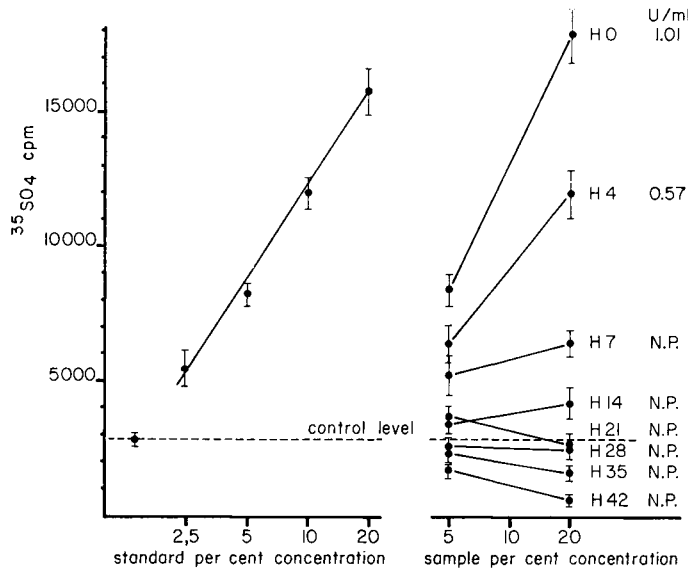


FIG. 2. — *Typical uptake pattern of a 180-day old hypox rabbit.* The number after H indicates the time-course after hypophysectomy (e.g. H 4 = 4 days after hypox). When possible, calculated potency is shown in U/ml on the right side of the figure. N. P. = no parallelism ; between H 7 and H 42 a single N. P. does not express all the changes occurring in incorporation levels and slope inversion.

In 40-day old hypox rabbits, Sm-A bioactivity changed only slightly during survival (fig. 3). At the end of the first post-operative week, sulphation activity fell moderately, then remained rather constant and positive. Before hypox, Sm-A bioactivities ranged between 0.31 and 0.81 U/ml. Sometimes just before death some sera inhibited the basal activity of the incubated cartilage.

In 180-day old hypox rabbits, the pattern of Sm-A bioactivity was quite different (fig. 4). Just before hypophysectomy Sm bioactivities ranged between 0.65 and 1.44U/ml. At 7 post-operative days, bioactivity dropped sharply (0.10 to 0.47 U/ml ; after 14 days, all the sera were inhibitory and remained so.

The difference in the Sm bioactivity of 40 and 180-day old hypox rabbits is shown in figure 5 which represents the average patterns of the two groups. Owing to the decreasing number of experimental animals with time, the standard error is not shown. Based on activity clearance rate over the first few days following hypophysectomy, Sm half-life would be about 4 days.

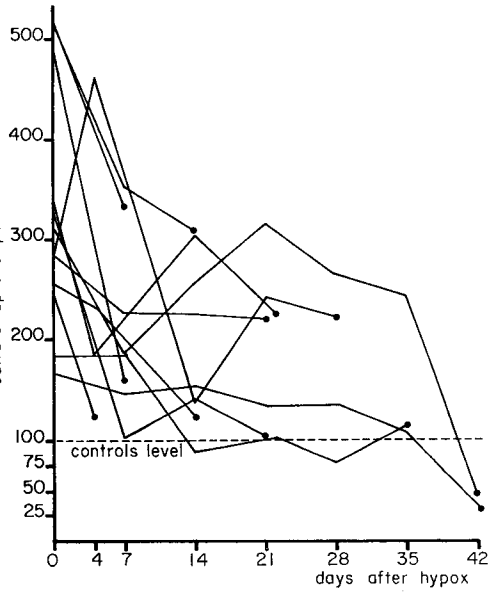


FIG. 3. — Hypophysectomy of 40-day old rabbits.

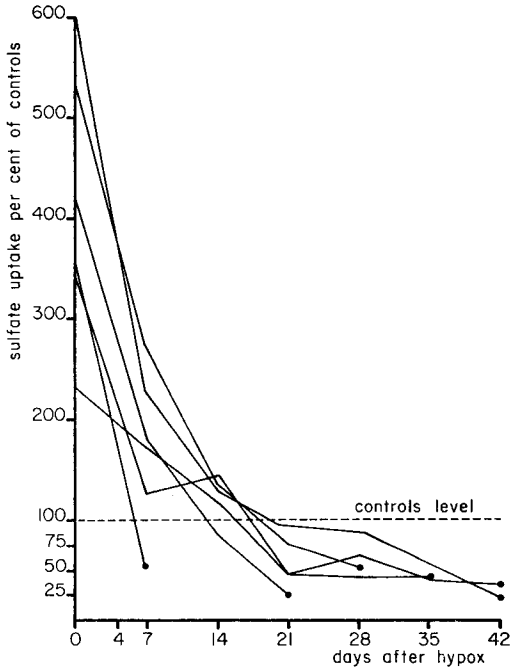


FIG. 4. — Hypophysectomy of 180-day old rabbits.

FIG. 3 and 4. — *Changes in pelvic $^{35}SO_4$ uptake (Sm-A bioactivity) produced by serum of young or adult rabbits in relation to time-course after hypophysectomy.* DO is the uptake level before anesthesia on the operative day. Radio-sulfate uptake for a final serum concentration of 20 p. 100 is expressed as a percentage of the incorporation by the controls in buffer. Each line shows the activity pattern of one rabbit, and the black points at the ends of the lines indicate the last sampling before death.

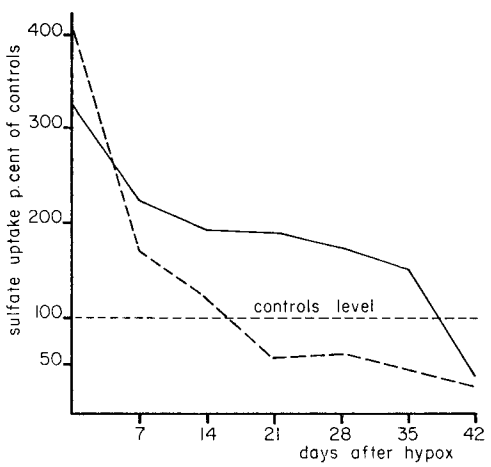
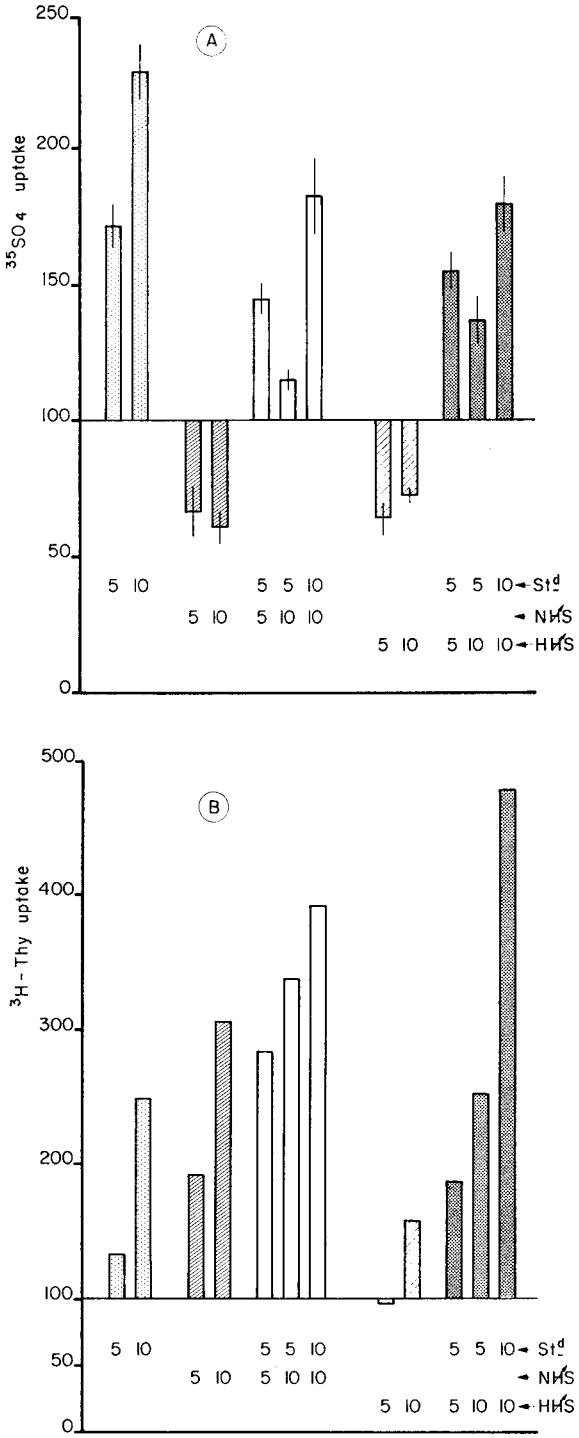


FIG. 5. — *Mean curves of figures 3 and 4 show* the differences between the two age groups. Because of the decreasing number of live rabbits, the standard errors are not given.


A
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$^{35}SO_4$ uptake
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5 10 → HHS
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^{3}H - Thy uptake
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5 5 10 → Std
→ NHS
5 10 → HHS

Heating at 100 °C for 15 min at pH 5.5 did not suppress the inhibitory effect on sulphate uptake, nor on basal cartilage activity or on the normal stimulatory activity of standard serum (fig. 6*a*). Two of the five hypox sera heated to suppress the inhibitor were studied with a double labelling technique. The results were quite different from those obtained with sulphate : the same sera which inhibited sulphation were stimulators of DNA synthesis (fig. 6*b*). Moreover, thymidine uptake was greatly diminished after heating (fig. 6*b*). To clearly represent the dissociation of the two effects, figure 7 shows the uptake patterns of both radioisotopes, using the serum of one male rabbit 42 days after hypophysectomy.

Discussion.

Salmon and Daughaday (1957) studying the sulphation factor already noted that hypophysectomy in 21-day old rats induced a decrease in serum sulphation activity. However, they specified that ^{35}S uptake into hypox rat costal cartilage, when stimulated with that serum, was higher than the controls. Later, Daughaday and Reeder (1966) reported that hypox rat serum often induced a lower ^{3}H-thymidine uptake in hypox rat cartilage than that obtained with the buffer alone. Therefore, hypox rat serum seemed to be truly inhibitory. In 1973, Salmon made the same observation concerning $^{35}SO_4$ uptake, and specified that this inhibitor, destroyed by trypsin, might be of peptidic nature.

In our rabbit strain, the linear growth of males lasts until week 15 after birth and from week 25 reaches a plateau. During this period (from 10 to 180 days), Sm-A bioactivity rises slowly from a mean of 0.5 U/ml to a mean of 1.0 U/ml, but with wide individual variations (Charrier, 1978). Our present results indicate that rabbit serum Sm-A bioactivity after hypophysectomy depends upon the age at surgery and, surprisingly, that significant sulphation activity is concomitant with continued growth, in spite of the fact that the young hypox animals have no pituitary. In 180-day old operated rabbits, Sm-A became nil 1 to 2 weeks after the operation, and the serum was inhibitory later. Since the standard and the sample were no longer parallel,

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FIG. 6. — *$^{35}SO_4$ (A) or ^{3}H-thymidine (B) uptake in chick embryo pelvis stimulated by the different sera.*

Normal standard rabbit serum ; Native hypox serum ; Standard + native hypox serum ; Heated hypox serum ; Standard + heated hypox serum.

Incorporation is expressed as a percentage of the controls without serum. Serum concentrations are indicated on the three lower lines. The total serum concentration of one column is calculated by adding the two concentrations comprising it. Example : in the next to last column, St^{d} → 5 and H → 10 = a final concentration of 15 p. 100, constituted with 5 p. 100 of standard serum and 10 p. 100 of heated hypox serum.

A : mean of 5 hypox adult rabbits ± standard error.

B : mean of 2 hypox adult rabbits.

owing to an inhibitor (fig. 2), inhibition via direct inactivation of serum somatomedin would seem to be excluded. However, the drop in activity in hypox growing rabbits at 40 days, when ponderal growth rate is maximum (Cantier *et al.*, 1969 ; Beaton, 1976), was much less evident and the serum remained stimulatory. The latter author reported that in two 50 to 60-day old rabbits, serum sulphation activity dropped with the porcine assay from 0.36 and 0.48 to 0.21 and 0.14 U/ml, respectively, 10 days after hypophysectomy. These results agree with the data of Salmon and Daughaday (1957, see above) and those of Vezinhet (1968) who failed to obtain any growth arrest in hypophysectomized rabbits less than 100 days old. Goussopoulos (1978) also noted that muscular growth stopped at 15 weeks in chickens hypophysectomized when 7 weeks old, but that muscular growth arrest was immediate when the animals were hypophysectomized after 15 weeks of age. Likewise, the ponderal growth of rats does not stop when hypophysectomy is performed before 4 weeks of age (Walker *et al.*, 1950). With a more accurate technique using oxytetracycline as an intravital marker, Thorngren *et al.* (1973) even estimated that longitudinal bone growth in the rat continued until 60 days, if hypophysectomy was performed before that age. Vezinhet (1973) studying ovines, noted a slight but significant bone weight increase after hypophysectomy in 25-day old lambs, but none in 100-day old ones. Studying the guinea-pig, Mitchell, Guillemin and Selye (1954) and Knobil and Greep (1959) reported that hypophysectomy did not affect body development.

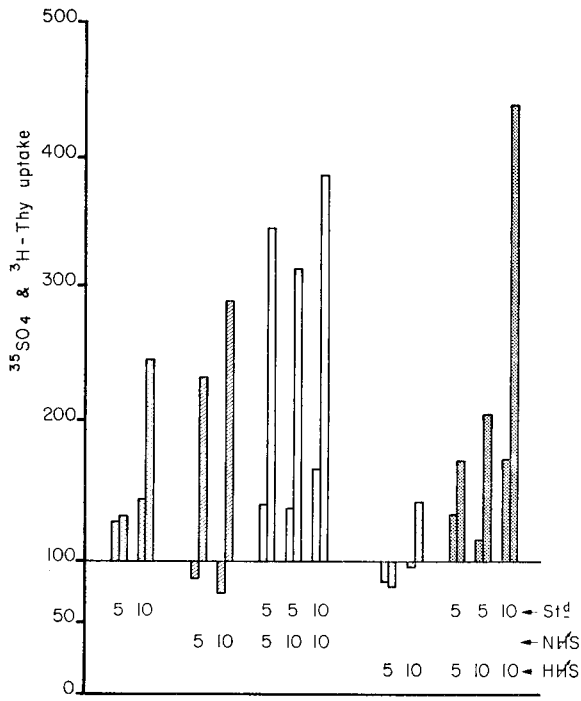

FIG. 7. — *Simultaneous incorporation of $^{35}SO_4$* (bars on left) *and ^{3}H-thymidine* (bars on right) *induced by the serum of one adult male rabbit 42 days after hypophysectomy*, expressed as a percentage of the controls. For a more complete explanation, see legend of figure 6.

These data support the argument that serum sulphation activity might not be completely GH-dependent. Several clinical observations suggest that under certain physiological conditions, plasma Sm levels are not correlated to GH levels. In obesity Sm levels are normal or elevated, although spontaneous or provoked GH secretion is lowered. On the other hand, in the case of food deprivation, plasma Sm activity decreases, although the GH secretion is higher than under normal conditions (Van den Brande and du Caju, 1973).

In a recent work, Reyne, Vezinhet and Prud'hon (1980) reported some changes in the alimentary behavior of the rabbit after hypophysectomy : a 15 p. 100 decrease in food intake when the operation was performed at 45 days, and a 30 to 50 p. 100 one when it was done at 180 days. Water consumption was always much higher in the days following the operation, then dropped to the same values as in the normals. Wether such a reduced food allowance could provoke a subsequent decrease in Sm bioactivity has not yet been studied. But, even if it could explain some of the differences between 40 and 180-day old rabbits, it is questionable whether it could account for all of them. This point needs further investigation.

Prolactin does, to some extent, stimulate Sm production (Francis and Hill, 1975), but after hypophysectomy all pituitary hormone influences are eliminated. However, in the absence of radioimmunoassayable GH, Sm activity may be present in the serum of patients with craniopharyngioma (Finkelstein *et al.*, 1972 ; Costin *et al.*, 1976). On the other hand, insulin growth factor (ex-NSiLA) exerts a marked growth-promoting effect (Morell and Froesch, 1973) and is a potent sulphation factor (Zingg and Froesch, 1973 ; Froesch *et al.*, 1976) ; its production in the dog does not seem to be highly pituitary-dependent (Eigenmann *et al.*, 1977). Thus, in young hypox rabbit serum the maintenance of some sulphation activity could be explained by continued IGF action. Insulin might also play an important role in Sm production. Daughaday and coworkers (1975, 1976*a*, *b*) induced Sm production in an isolated rat liver perfusion system by adding only insulin into the perfusion liquid. Identical results have been reported by Shapiro, Waligora and Pimstone (1978). Thus, the remaining Sm-A bioactivity found in 40-day old hypox rabbit serum might be due to the action of the serum's own insulin or IGF. How, then, can we explain the clearance of all Sm bioactivity from the serum of 180-day old rabbits since this serum not only loses its stimulatory ability but becomes inhibitory ? There must be one or more factors in the serum which are not normally there, or which are enriched under abnormal physiological conditions. The pituitary would play an inhibitory role in the synthesis of these factors, and the suppression of the pituitary brake would allow their full expression. The long time-course required for inhibitory activity to manifest itself suggests the progressive establishment of several intermediate mechanisms.

In normal conditions, rat Sm half-life would be about 3 to 4 hrs (Daughaday *et al.*, 1968 ; Cohen and Nissley, 1976). However, the half-life of free Sm (about 8 min) differs from that of Sm bound to its carrier protein (3 to 4 hrs) (Cohen and Nissley 1976). So after hypophysectomy, Sm half-life should be shorter because of the progressive clearance of the binding protein. On the contrary, Almqvist and Falkheden (1961) using bioassay reported the half-life of Sm in hypophysectomized humans as 9 to 18 hrs, while Takano *et al.* (1977) using radioreceptorassay observed a half-life of 24 hrs. Even with a 24-hr half-life, it is easy to calculate that, 7 days after hypophysec-

tomy, Sm activity was less than 1 p. 100 of its initial level : the initial Sm activity of our rabbits ranged from 0.65 to 1.44 U/ml, 7 days after hypox it ranged from 0.10 to 0.47 U/ml. If sulphation activity was due solely to somatomedin, our results would suggest that Sm-A half-life in the adult hypox rabbit would be much longer than in humans under the same experimental conditions, i.e. about 4 days. But we know that other factors may play a role in sulphation activity (see above), and thus we cannot draw conclusions on Sm half-life in rabbits only on the basis of the clearance rate of serum Sm bioactivity ; further studies using exogenous Sm injections are needed.

The nature of the inhibitor occurring in serum under certain conditions has not yet been well characterized. Hypox rat serum inhibitor is destroyed by heating or trypsin, suggesting a proteic structure (Salmon, 1973). The inhibitor we found in newborn rabbit serum was also heat labile (Charrier, 1978), but that in adult hypox rabbit serum, acting on sulphate uptake, is not abolished by heating, indicating that it might be different. Furthermore, the data obtained with double labelling seemed to evidence that sulphation and thymidine activities were due to separate factors. A large part of the thymidine factor would escape from sulphation inhibitor influence, and would be thermosensitive or co-precipitated with protein when heated. In contrast, sulphation activity is reputed not to be thermolabile, and so is the sulphation inhibitor of hypox rabbit serum (fig. 5*a*). A thymidine inhibitor has been shown to exist in the hypox rat (Daughaday and Reeder, 1966), and in the newborn rabbit we attributed this role to a common somatomedin inhibitor (Charrier, 1978). In the light of the present results, we do not think that SO_4 and thymidine inhibitions are due to the same factor.

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Résumé. Le dosage biologique de Hall (1970) par incorporation *in vitro* de $^{35}SO_4$ dans le cartilage d'embryon de poulet a été utilisé pour suivre l'évolution de l'activité somatomédine (Sm) du sérum de lapin après hypophysectomie. Chez les animaux opérés à l'âge de 40 jours, l'activité Sm accuse une diminution sensible dans les jours qui suivent, et se stabilise à un niveau qui est loin d'être négligeable. Au contraire, chez les lapins hypophysectomisés à 180 jours, l'activité Sm chute rapidement, devient nulle, et au-delà de 14 jours environ les sérums deviennent même inhibiteurs. En se basant sur la vitesse moyenne de disparition de l'activité dans ce deuxième groupe d'animaux, on peut estimer la demi-vie apparente de Sm après hypophysectomie chez le Lapin à environ 4 jours. Cependant, bien qu'utilisé par d'autres auteurs, ce moyen d'estimation de la demi-vie est criticable (voir discussion) et doit donc être admis avec quelque réserve.

Le facteur inhibiteur qui apparaît n'a pu être éliminé par chauffage des sérums à 100 °C.

Par double marquage au $^{35}SO_4$ et à la thymidine tritiée, il s'avère que le facteur qui inhibe la sulfatation du cartilage n'inhibe pas l'incorporation de ^{3}H-Thy dans l'ADN. Comme par ailleurs il a été montré qu'il existait aussi un inhibiteur de l'incorporation de thymidine, il se pourrait que « sulphation factor » et « thymidine factor » soient des entités séparées, ayant chacune son propre inhibiteur. Ajoutons que dans le cas des lapins étudiés le « thymidine factor » subsiste après hypophysectomie, alors que le « sulphation factor » a disparu, et qu'il est en grande partie détruit par le chauffage.

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