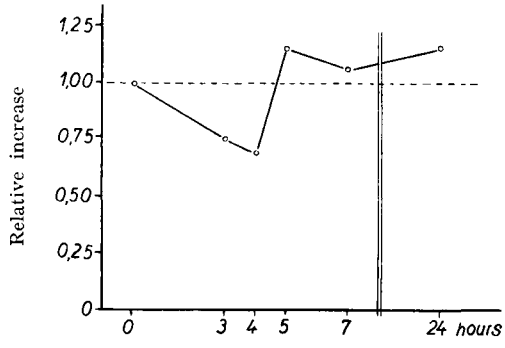
If the relative increases in cases with and without hyaluronidase are compared, it is seen that the former values are consistently higher than the latter. If the t-values (third row from the bottom) of the three groups of experiments are pooled, the difference in question becomes statistically significant ($P < 0{\cdot}001$). The pooling is effected by dividing the sum of the three t-values by $\sqrt{3}$ and using the resultant value as a t-value with infinite d. f.[1].



The relative increase in rats of the spots of India ink (0·1 ml containing 15 VRU hyaluronidase) given intradermally at different times after i. v. administration of 50 mg per kg. body weight of colloidal iron. The relative increase was calculated by comparison of the areas, immediately and 60 minutes after the injection of India ink, and by division of this value with the mean value from 6 control animals, which were injected with physiological saline solution instead of colloidal iron. Each circle represents 3 experiments.

Discussion. The RES may be said to consist of the lymphatically and the haemovascularly located phagocytic elements. If a colloid is injected intravenously, it is taken up by the haemovascularly located reticuloendothelial cells. One cannot find iron colloids in the lymphatic macrophages after intravenous iron injection (ANDERSSON and REXED[2]). In the above mentioned experiments the hyaluronidase effect was tested intradermally. It is of interest to notice that after intravenous blockade we obtained a marked inhibition of the spreading of India ink.

Using Ferrivenin the inhibiting effect in the blockaded animals lasted a relatively short time. After five hours we could no longer show any inhibition of the spreading of India ink. This result was similar to that which we obtained when we investigated the blocking effect of Ferrivenin with the aid of radioactive colloids. The phagocytic ability of the RES was resumed in a few hours.

We intend to make a further study of the RES and the spreading effect. E. R. GABRIELI[3] and M. CAMPANI

Department of Experimental Histology, Karolinska Institutet, Stockholm, April 2, 1951.

Zusammenfassung

Die intradermale Ausbreitung von chinesischer Tusche in Gegenwart von Hyaluronidase wird vermindert, wenn das retikuloendotheliale System mit kolloidalem Eisen (Ferrivenin) blockiert wird. Dieser Effekt ist größer, wenn Ferrivenin als blockierendes Agens intravenös anstatt intraperitoneal angewendet wird. Die Wirkung hält nur wenige Stunden an. Es wird auf die Ähnlichkeit verwiesen, die zwischen der Ausbildung der Blockade und der verminderten Ausbreitung der Tusche besteht.

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[3] Present adress: Section of Medical Physics, Yale University School of Medicine, New Haven, Conn.

Autoradiographic Analysis of the Distribution of Labelled Ca and P in Bones

A microphotographic-photometric study of the X-ray absorption of bone ground substance revealed that minerals are unevenly distributed in the latter[1]. Resorption and new formation of bone tissue determine changes in the distribution of minerals in compact and spongy bone. The Ca content in recently formed pieces of secondary bone (e.g. Haversian systems, *osteons*) is in general from 10 to 12% lower than the Ca content in the fragments of the older periosteal bone or in the interstitial remnants of previously built osteons; the mineralization of the newformed osteons undergoes a slow and progressive increase.

Autoradiographs made from ground sections of bones compacta of young dogs a few hours after administration of labelled P, showed a greater activity of the matrix in recently laid down osteons than in the older primary bone tissue; viz., a larger number of labelled atoms is incorporate in the ground substance of the relatively less calcified pieces of secondary bone.

However, it remained open to question whether the different deposition of labelled atoms in the various structures of bone depends on the different situation of the latter in regard to the blood vessels, or on different physico-chemical properties of the ground substance itself. It is obvious that mineral atoms of the blood can be adsorbed more easily in the ground substance which neighbors the vascular channels than in the matrix which lies at a greater distance from blood vessels, namely in the interstitial systems.

A different technique was applied in this research to obtain further information on the alternative mentioned.

Ground sections of fresh bone compacta from 25 to 45 micra in thickness were kept from a few hours to a few days in solutions of radioactive Calcium chloride[2]. The sections, thoroughly washed in distilled water and dried at room temperature, were pressed against the emulsion side of photographic plates or films (C2 Ilford Nuclear Research plates, Ferrania X-ray films)[3]. This procedure has the advantage that the radioactive Ca comes in ready and almost uniform contact with each single particle of the bone section; thus the possibility that a different deposition of labelled atoms in the various structures of bone might depend on the topography of the latter in regard to blood vessels was ruled out.

Figures 1 and 2 illustrate the Roentgenmicrophoto (negative) of a cross section of the diaphysis of the femur (man 47 years old) with its corresponding autoradiograph after treatment with labelled Ca. It is apparent (fig.2) that the radioactivity is not uniform throughout compacta. The comparison in the same section of the degree of radioactivity (fig. 2) with the degree of calcification of the various structures (fig. 1), demonstrates that a greater amount of radiocalcium is deposited in the ground substance of recently formed osteons; the Ca content of the latter is from 6 to 16% lower than that of the neighboring older bone tissue.

The distribution of Ca^{45} in the ground substance appears to be exactly the same as described above when

[1] R. AMPRINO and A. ENGSTRÖM, Boll. Soc. ital. Biol. sper. *26*, 148 (1950); Acta anat. (in press).
[2] The author wishes to express his thankfulness to Prof. P. LACROIX, Louvain, who kindly supplied the Calcium chloride for these experiments.
[3] Technical details and a complete description of results will be published elsewhere.

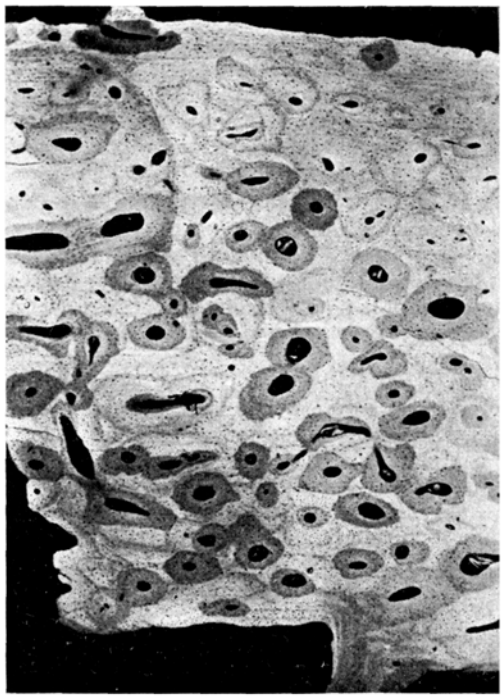

Fig. 1.

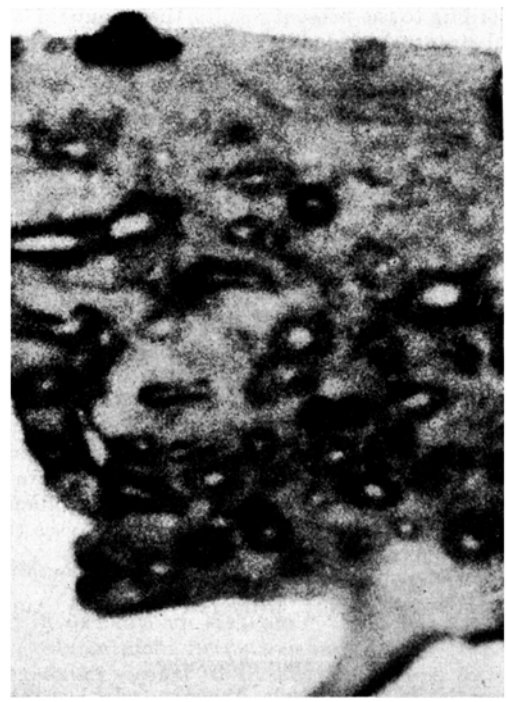

Fig. 2.

sections of bones, fixed and preserved in formalin (or ethanol) or sections of macerated bones from museum specimens, are treated with radioactive Calcium chloride. Autoradiographs of sections of bones fixed in formalin or ethanol and microincinerated for three hours at 700°C, still show slight differences in the uptake of labelled Ca into the various structures. Therefore, the different deposition of Ca^{45} in the various structures of bone compacta does not depend on quantitative differences of cell (or of enzyme) activity, but, partly at least, on differences of the proportion of organic to inorganic components of ground substance. The amount of Ca^{45} deposited seems to depend also on the proportion between osteomucoid and collagen of the matrix. As a matter of fact, the Ca^{45} uptake in sections of the same thickness is greater for bones of reptilians and of birds than for mammalian bones (cf. also the results of HEVESY *et al.*[1]); the deposition of Ca^{45} is relatively greater also in bones of young than of adult specimens of the same species (ox, man).

The deposition of P^{32} was tested on sections of bones contiguous to the ones which had been used for the study of Ca^{45} uptake. The distribution of P^{32} appeared to be the same as that of Ca^{45}; however, given the sections thickness, the definition of the autoradiographs was rather poor when P^{32} was used.

The fate of the atoms of radioactive elements and the mechanism of their fixation to bone matrix is still a matter of speculation[2]. It is obvious that labelled atoms can be incorporated into the ground substance which is progressively laid down[3]; however, the laying down of bone tissue is a slow process, while the uptake of labelled atoms occurs promptly[1]. It has been maintained for a long time that an exchange-reaction between the atoms of the fluid and of the solid phase takes place *in vitro* as well as *in vivo*. The superficial atoms of Ca of the apatite crystals could be replaced by atoms of Ca, U, Sr, etc. of the fluid phase[2]. Only 1/5 of the atoms of Ca of the crystals could thus be replaced[3]; according to HARRISON and HARRISON[4], the whole Ca of apatite could be progressively substituted by the labelled Ca of the body fluids.

The present research shows that the uptake of labelled Ca and P is remarkably greater in the more recently built and not yet fully mineralized structures than in the more calcified portions of bone. The different uptake might depend on a different rate of the exchange-adsorption of mineral atoms in the various structures of bone; or possibly, only a part of the atoms which are deposited in the recently laid down and less calcified structures exchange with pre-existing ones of the solid phase. Some might become progressively fixed to the already calcified matrix without liberation of atoms from the apatite crystals, viz., an actual *addition* of mineral atoms might occur. The latter alternative seems to be in good agreement with the progressive calcification of secondary bone as demonstrated by X-ray absorption analysis[5].

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According to the present results, the amount of labelled mineral atoms which can be bound to the already calcified ground substance of a given bone seems to depend to a great extent on the relative amount of recently built and not yet fully calcified pieces of ground substance. Evidently the number of pieces of recently laid down bone tissue is greater the higher the rate of microscopic rebuilding. Thus, the activity of bones after treatment with radioactive elements should be greater the wider the reconstructive processes which occur in spongy and compact bone[1]. In this respect, the differences of activity of the skeleton of young and of mature animals of the same species after administration of labelled Ca or P or of U, Sr, etc.[2], might depend—at least partly—on the well-known differences of the rebuilding rate of the skeleton in the various stages of life[3].

The same reasoning might provide an explanation of the differences of radioactivity between diaphysis and epiphyses of long bones (cf. *i.al.*[4]); indeed, the rate of the microscopic reconstruction is higher throughout life in spongy bone of epiphyses and of metaphyses than in compact bone of diaphysis[5].

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Zusammenfassung

Dünnschliffe von frisch fixierten oder mazerierten Knochen wurden *in vitro* der Einwirkung von radioaktiven Ca- und P-Salzen unterworfen. Die Autoradiographien beweisen, daß Ca^{45} und P^{32} in größerer Menge von den verhältnismäßig weniger kalzifizierten Abschnitten der Grundsubstanz des Knochens übernommen werden. Die Menge der anorganischen Salze, welche unter diesen Bedingungen von den verschiedenen Knochen aufgenommen wird, hängt hauptsächlich von der relativen Menge der vorhandenen organischen Substanzen und von den in der Grundsubstanz enthaltenen Osteomukoiden ab.

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L'utilisation du chlorure de 2,3,5-triphényltétrazolium pour l'étude de l'activité déshydrogénasique des mitochondries

Le chlorure de 2,3,5-triphényltétrazolium (TPT), obtenu par VON PECHMAN et RUNGE[1], est un dérivé incolore, très soluble dans l'eau, qui, sous l'action des réducteurs – inorganiques (hydrosulfite de sodium, sulfure d'ammonium, etc.) ou organiques – se transforme en un corps fortement coloré en rouge, insoluble dans l'eau, soluble dans de nombreux solvants organiques, le triphénylformazan. Nous nous trouvons donc en présence d'un système dont la forme colorée est représentée par l'état réduit, fait particulièrement remarquable pour son utilisation en vue de la détermination des déshydrogénases.

Nous avons utilisé la méthode de GREEN (homogénéisation au mixeur, centrifugation dans une solution de KCl à 9‰)[1] pour la préparation de mitochondries (cyclophorase) de foie et de rein de rat et de lapin. La technique adoptée pour l'étude de l'activité déshydrogénasique est la suivante: à une quantité donnée de suspension de mitochondries (1 cm³), on ajoute un tampon phosphate 0,2 M à pH 7,3 (0,5 cm³), le substrat correspondant à l'enzyme à étudier et des activateurs variables selon les cas ($MgCl_2$, adénosinetriphosphate, etc.). Après avoir complété à un volume donné (3 cm³) par de l'eau bidistillée, on ajoute 1 cm³ d'une solution aqueuse de TPT à 0,1%. Il est indispensable d'utiliser une solution fraîchement préparée de TPT et maintenue à l'abri de la lumière. En effet, le TPT, très stable à l'état anhydre, s'altère en solution aqueuse sous l'action de la lumière qui, par une réaction d'oxydoréduction interne, le transforme en triphénylformazan et en chlorure de 2,3-(*o*-biphénylène)-5-phényltétrazolium[2]. On porte au bain-marie à 37°C pendant 20 min et on extrait alors le formazan.

L'extraction de la totalité du colorant est difficile en raison de l'adsorption d'une partie de celui-ci sur les mitochondries. L'adjonction d'acides ou de bases forts décolore le formazan et est donc à rejeter. L'acétone (6 cm³) permet une extraction satisfaisante, à condition d'agiter fortement; l'adjonction d'acide trichloracétique (1 cm³ à 40%)[3] n'est pas utilisable pour l'extraction du triphénylformazan, car elle décolore celui-ci. Les détergents (zéphyrol) ne facilitent pas l'extraction. En dehors de l'acétone, nous avons obtenu une excellente extraction par le mélange suivant: alcool éthylique, 5 cm³ + alcool nonylique (ou caprylique), 1 cm³.

Après adjonction du solvant, on agite, centrifuge, filtre et mesure l'absorption du filtrat à 492 mμ. Les lectures ont été faites à cette longueur d'onde à l'aide d'un spectrophotomètre de Beckman, modèle DU. Nous avons déterminé la courbe d'absorption du TPT et du triphénylformazan et trouvé qu'alors que le premier de ces corps n'absorbe pas dans le visible, le second (dissous dans un solvant organique, l'acétone par exemple) absorbe fortement entre 400 et 550 mμ, avec un maximum à 492 mμ ($\varepsilon \times 10^{-3} = 14{,}9$). Dans l'ultraviolet, TPT et formazan (ce dernier dissous dans l'éther et non dans l'acétone vu la forte absorption de ce solvant) absorbent fortement au-dessous de 310 mμ, avec un maximum à 245 mμ pour le TPT et 295 mμ pour le formazan. Rejoignant les conclusions de KUN et ABOOD[4], nous avons d'autre part établi que la densité optique des solutions de formazan est bien une fonction linéaire de la concentration, aussi bien dans le visible que dans l'ultraviolet. Ce fait permet donc aisément la détermination spectrophotométrique de la concentration en formazan.

Les lectures d'absorption doivent être faites peu de temps après la fin de la réaction. En effet, si le formazan est relativement stable en solution dans l'acétone ou

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