ing out more than speculative analysis, but certainly, as suggested by Lars Onsager, there must be a momentum deficiency in the microdomain of the pore in the solution side of the barrier. That is, in a solid region of the barrier, the time average transfer of momentum is that prescribed by the hydrostatic pressure of the phase, but in the opening of the pore there is a deficiency since the momentum arising from the macromolecule is not transferred to the solvent species in the pore, being cut off by the finite size of the pore. Thus, within the pore and only within the pore, a gradient of pressure arises and quasi-laminar flow ensues from the solvent side to the solution.

Although the experimental observations in the osmometer experiment do not demonstrate the diffusion component of flux explicitly, it is reasonable to assume that this component is present:

$$\left(\frac{dn}{dt}\right)_{diff.} = \frac{-DA}{RT} C \frac{\Delta u}{\Delta X} \cdot$$

$$= \frac{DA}{RT} \frac{C}{\Delta X} [\bar{V}\Delta P + \Delta RT \ln N]$$

$$= \frac{DA}{RT} \frac{C\bar{V}}{\Delta X} [\Delta P + \frac{RT}{\bar{V}} \ln (1 - N_{H_2O})]$$

$$= \frac{DA}{RT} \frac{1}{\Delta X} [\Delta P - \pi]$$

Thus, the total flux is

$$\frac{dn}{dt} = \left[\frac{DA}{RT\Delta X} + K f(A)\right] [\Delta P - \pi]$$

The diffusion component would be all-important in a barrier whose "pores" have a cross-sectional area of the order of the solvent molecules such that only a molecular-molecular drift of the solvent could occur.

In conclusion, the point to be emphasized for workers in the field of membrane permeability is the fact that in osmotic transfer the chemical potential difference of the solvent can give rise to both a quasi-laminar flux and to a diffusion flux, the relative importance of the two components being dependent on the nature of the barrier. For most barriers, the predominant component is the quasi-laminar flux.

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3 May 1957

## Aggressive Behavior in Castrated Starlings

Androgens have long been known to affect the aggressive behavior of birds and mammals. Experiments conducted during the last two decades have shown that animals of various species ceased aggressive behavior when they were castrated, or rose in social rank when they were given injections of testosterone. This paper (*1*) reports the maintenance of aggressive behavior in castrated starlings and the failure of testosterone to affect their social rank.

The methods consisted of observing castrated starlings (*Sturnus vulgaris*) that were living in a large room (14 by 16 feet). Eleven birds were bilaterally castrated on 20 Dec. 1956, when the testes were still in the regressed winter condition but were starting to increase. The birds were painted on the tail with bright colors for individual identification. These birds maintained fighting and singing behavior for a month. A conventional diagram describing the social rank was prepared. In most cases the relative position was clear, but in some cases the birds may have been tied for position, and in other cases no contests were observed.

On 15 Jan. a series of injections of graded doses of testosterone was begun, to determine the effect of testosterone on the seminal vesicle (*2*). The dosage was not known to the observer. The rank of the individuals did not change during a period of 10 days. The birds that were injected with control material remained in their rank. The birds that received the highest amounts of testosterone were sixth and ninth in rank even at the end of the 10-day period. It was suspected that three of the birds might have some testicular tissue because their bills remained yellow. These birds ranked first, third, and eighth and, on autopsy, were found to have some tissue.

Because these results were the gleanings from another experiment, a program was specifically planned. Five birds were castrated 2 Feb. and were observed until 11 Mar. A rank was obvious, and song continued vigorously. Injections of testosterone (begun 11 Mar.) at various dosages had no effect on rank. On autopsy, on 21 Mar., one bird (second in rank) had 25 mg of testicular issue, but the top-ranking bird had none.

These results demonstrate that castrated male starlings maintain a rank, as do normal birds. Since the aggressiveness of these adults might be the result of learning, experiments with young birds are planned. However, the aggressiveness might result from androgen from another source. But the threshold of response would have to be below the level that controls bill color and growth of seminal vesicles because castrated birds have black bills and minute seminal vesicles. Furthermore, the fact that injections of large amounts of testosterone did not alter rank indicates that androgens are not involved. The aggressiveness might be responsive to another hormone, such as a hypophyseal hormone, since Witschi (*2*) concluded that, in some birds, plumage changes are controlled by gonadotropins. This possibility is being explored.

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## Nature of Fluorophore Localizing in Tetracycline-Treated Mouse Tumor

It has been previously observed that certain chemical agents such as fluorescein (*1*) and hematoporphyrin (*2*), when administered parenterally to tumor-bearing animals, tend to localize in the tumor tissue. This phenomenon finds limited clinical applications in the localization and diagnosis of neoplastic diseases (*3*). The fluorophore in the tumor tissue was usually assumed to be the unchanged compound administered, without, however, inquiry being made into its exact chemical nature.

Recently, Rall *et al.* (*4*) reported that, in animals bearing transplantable tumors, localized fluorescence was noted in the bones and the tumor tissue after treatment with any of the tetracyclines. The discovery aroused considerable interest in that a variety of animal tumors as well as a few human neoplasms exhibited this behavior. In addition, the localized fluorescence persisted as long as the animals survived (*5*).

In view of the sustained interest in the problem and the obvious chemotherapeutic possibilities implied, an effort has been made in our laboratory to study the chemistry of the fluorophore in the tetracycline-treated mouse tumor. In this report, evidence is presented to show that the localized fluorescence is attributable to unchanged tetracycline which, however, probably does not exist as such in the tumor tissue, but rather as a loose complex bound with a peptide which is one of the normal constituents of mouse sarcoma tissue.

$CAF_1$ mice weighing 20 to 24 g with 6-day-old sarcoma S-37 were injected in-

traperitoneally with tetracycline hydrochloride (*6*) (3 mg in 0.3 ml of saline). Following three such daily treatments, the mice were sacrificed on the third day after the last injection, and, under ultraviolet illumination (*7*), the fluorescent parts of the tumor were excised. The tumor tissues collected were homogenized in water, about 2 ml/g of tissue, and the homogenate was dialyzed in cellulose casing (*8*) with shaking against dilute hydrochloric acid (0.1*N*, about 8 ml for every gram of tissue) for 2 hours (*9*). The dehydration of tetracycline to anhydrotetracycline (*10*) is very fast in acid stronger than 0.1*N*; nevertheless, in 0.1*N* hydrochloric acid, tetracycline is stable for at least 4 hours. When the *p*H of the filtered dialyzate was adjusted to above 7 by the addition of sodium hydroxide, the fluorophore inevitably came down as a flocculent precipitate which fluoresced bright greenish yellow under ultraviolet light. Under identical experimental conditions, from the dialyzate of the tumor tissue of control mice that received no tetracycline, there was likewise obtained a flocculent precipitate which was, however, nonfluorescent. In both cases, the precipitates gave positive ninhydrin and biuret tests, thus suggesting their peptide nature. For the electrophoresis and paper chromatography experiments described in the following paragraphs, a more concentrated solution of the fluorophore (and similarly of the peptide in untreated mouse tumor) could be conveniently prepared by first separating the precipitate by centrifugation, then washing with a little water, following with centrifugation again, and finally dissolving in a minimum amount of 0.1*N* hydrochloric acid.

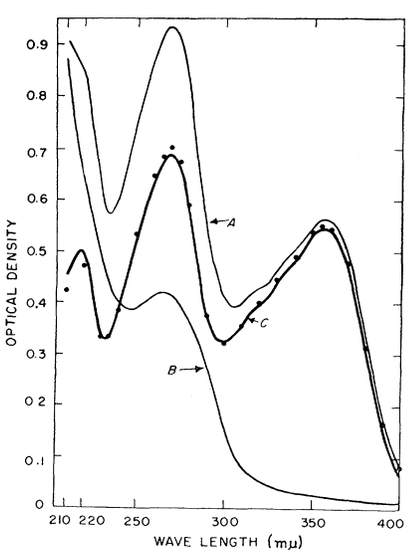


Fig. 1. Spectra in 0.1*N* HCl of (*A*) the fluorophore, (*B*) the nonfluorescent precipitate from control tumor, and (*C*) tetracycline (18 μg/ml). The closed circles are the calculated optical densities due to the prosthetic group of the fluorophore.

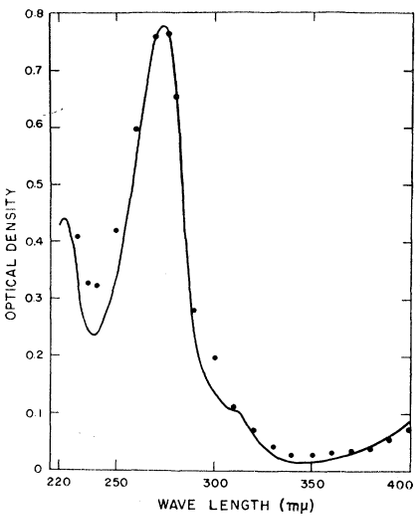


Fig. 2. Spectrum of anhydrotetracycline (6.7 μg/ml in 1*N* HCl). The closed circles denote the calculated optical densities due to the altered prosthetic group of the tetracycline.

At *p*H higher than 7, after saturation of the alkaline dialyzate with salt, the fluorophore could be partially extracted into butanol. Examination of the ultraviolet absorption spectrum of the butanol extract against a similar extract of the control tumor tissue as blank revealed that, compared with the spectrum of tetracycline itself in butanol, the $\lambda_{max.}$ at 367 mμ of the parent compound had undergone a shift of 15 mμ to 382 mμ (*11*, *12*). Such a bathochromic shift—analogous to, for example, that observed of chlorophyll in plant cells (*13*)—could be ascribed to the formation of a complex between tetracycline and the peptide in tumor tissue. This view receives further support from the results of paper-electrophoresis and paper-chromatography experiments. In the former experiments, using the Spinco model R electrophoresis cell of the Durrum type, at a constant voltage of 250 in *p*H 8.6 barbiturate buffer, the migration rate of the fluorophore differed quite noticeably from that of tetracycline. As for the paper-chromatography work, the sodium arsenite system, with ascending flow (*14*), was employed. The $R_f$ value for the fluorophore was observed to be about 0.10 as against 0.16 for tetracycline.

However, in weak acid—for example, 0.1*N* hydrochloric acid, a comparison of the ultraviolet spectrum of the fluorophore with that of tetracycline and also of the nonfluorescent precipitate from control mouse tumor displayed no new absorption bands, nor did it display any shift of the existing bands (Fig. 1). It seems safe, therefore, to conclude that the complex formed between tetracycline and the peptide must be a loose one; in fact, the complex readily dissociates in acid. Assuming that the peptide of the control and that of the tetracycline-treated tumor exhibit identical ultraviolet-absorption patterns, it is easy to calculate the absorbance owing to the prosthetic group of the fluorophore at various wavelengths by simply deducting the corresponding absorbance owing to the peptide from the total absorbance. The reasonableness of the assumption at once becomes evident; through the outcome of the calculations, an absorption curve of the prosthetic group can be constructed, which is exactly identical with the spectrum of tetracycline at 18 μg/ml in 0.1*N* hydrochloric acid (Fig. 1).

The identification of the prosthetic group of the fluorophore with tetracycline is further strengthened by the observation that, as a result of the exposure to 1*N* hydrochloric acid for 1 hour or longer, the fluorophore disclosed profound changes in its ultraviolet absorption spectrum. By applying the same mathematical technique, we calculated the absorbance contributed by the altered prosthetic group and plotted it against the wavelength (Fig. 2). For all practical purposes, the absorption curve represents nothing but the spectrum of anhydrotetracycline. This is hardly surprising, for it is known that the conversion of tetracycline into the anhydro-compound proceeds with rapidity in acid.

Thus, without actually isolating the prosthetic group, it has been possible to demonstrate that the fluorophore in the tetracycline-treated mouse tumor is most likely a complex formed of the parent compound and a peptide. This complex dissociates readily into its components in acidic media. At present, the mechanism for the localization and persistence of the tetracyclines in the tumor tissue remains unclear. Work is currently in progress in our laboratory on the metabolism of the tetracyclines and also on other compounds closely related to the tetracyclines that might manifest this phenomenon. The chemistry of the peptide associated with tetracycline is being actively investigated (*15*).

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7 May 1957

## Prevention of Toxicity of Amethopterin for Sarcoma-180 Cells in Tissue Culture

The present paper is a preliminary report on the finding that sarcoma-180 (S-180) cells grow normally when the function of folic acid is prevented by amethopterin (Methotrexate), if the medium is supplemented with products of the biosynthetic reactions dependent on folic acid cofactors. It is known that certain microorganisms which require folic acid for growth—for example, *Streptococcus faecalis* 8043—can grow in the absence of this vitamin in a medium containing thymine and adenine or guanine (*1*). That folic acid is one of the nutritional requirements for the growth of mammalian cells in tissue culture was shown by Eagle (*2*). The inhibition of the growth of sarcoma-180 cells in such a medium by amethopterin has been demonstrated (*3*).

The techniques of Eagle (*2, 4*) were used. The medium contained 5 percent thoroughly dialyzed horse serum. The cells were grown in the experimental media for 7 days. The growth of the cells in the presence of amethopterin in a medium containing hypoxanthine, thymidine, and glycine is shown in Table 1. Disintegration of the cells occurred if hypoxanthine or thymidine was omitted from the mixture. In the absence of glycine, some growth was observed, indicating either a small amount derived from the dialyzed horse serum or some formation of glycine, possibly from the exogenous L-threonine (*5*). When glycine was added to this medium, the growth was comparable to that of the control. The complete mixture fully supported the growth of sarcoma-180 cells even in the presence of amethopterin at concentrations 10,000 times that ordinarily required for complete inhibition (Table 1). In body fluids, the concentrations of such compounds could be critical to the effectiveness of amethopterin on neoplastic cells *in vivo*. Similar compounds in the crude medium (chicken plasma clot) might also explain the failure of amethopterin to inhibit sarcoma-180 cells, as reported by Biesele (*6*).

When the function of folic acid was prevented by amethopterin, it was found that sarcoma-180 cells were able to utilize adenine, adenosine, deoxyadenosine, hypoxanthine, and inosine equally well; guanosine supported slower growth of sarcoma-180 cells under these conditions; that is, there was only a threefold increase in 7 days, whereas xanthine and xanthosine were inactive. These results indicate that some "adenine" was derived from guanosine, but the extent to which "guanine" or "adenine," or both, were derived from xanthine and xanthosine was insignificant.

In the presence of amethopterin, thymidine, and glycine, the cells disintegrate if purines are not supplied in the medium (Table 1). Under such conditions it is unlikely that purine synthesis *de novo* occurs. Thus, the single purine in the medium must serve as the sole source, not only of adenine and guanine of nucleic acids, but also of all the coenzymes containing purines as structural constituents. Accordingly, this technique appears to be useful for the study of the pathways of purine metabolism. The present work demonstrates for the first time that mammalian cells (sarcoma-180) are fully capable of using exogenous purines for growth and multiplication.

Further work demonstrated that thymidine could be replaced by thymidylic acid for the growth of sarcoma-180 cells in the presence of amethopterin, glycine, and a purine, but thymine and thymine-riboside had no activity in this respect. A mixture of thymine and deoxyadenosine did not replace a mixture of thymidine and adenine, indicating that this type of transdeoxyribosidation did not occur.

It is seen that the presence of amethopterin creates new requirements for the growth of the tumor cells *in vitro*. When these requirements are met by preformed purines, thymidine, and glycine, inhibition of growth might still be achieved if the utilization of even one of these compounds were prevented. Logical combinations for chemotherapy are thus suggested. The new requirements created by amethopterin probably differ in different species and tissues. It is already known that differences exist in the abilities of various tissues and species to utilize preformed purines *in vivo* (*7*). In addition, rabbit fibroblasts, unlike sarcoma-180 cells, require exogenous L-serine for growth in tissue culture (*8*).

Table 1. Growth of sarcoma-180 cells in tissue culture in the presence of amethopterin. Folic acid (pteroylglutamic acid) was present at a concentration of $2 \times 10^{-7} M$.

| Varied supplements in the medium | | | | Degree of cellular multiplication† |
|---|---|---|---|---|
| Amethopterin* ($M$) | Hypoxanthine $3 \times 10^{-5} M$ | Thymidine $3 \times 10^{-5} M$ | Glycine $1 \times 10^{-4} M$ | |
| 0 | | | | 5.7‡ |
| $3 \times 10^{-8}$ | | | | 0.70 |
| $3 \times 10^{-7}$ | | | | 0.34 |
| $3 \times 10^{-7}$ | + | + | + | 5.7§ |
| $3 \times 10^{-4}$ | + | + | + | 6.1 |
| $3 \times 10^{-7}$ | | + | + | 0.60 |
| $3 \times 10^{-7}$ | + | | + | 0.43 |
| $3 \times 10^{-7}$ | + | + | | 1.8 |

* 4-Amino-10-methyl-pteroylglutamic acid.
† Referred to inoculum as 1; determined by the method of Oyama and Eagle (*11*); correlation of the protein determinations with cell counts is discussed by Oyama and Eagle (*11*).
‡ Control.
§ The culture has been carried for 3 weeks under these conditions and is being maintained.

Thymine or thymidine increased the rate of the development of amethopterin resistance in *Streptococcus faecalis* 8043 (*9*). The effect of similar factors on the development of amethopterin resistance in mammalian cells is under study (*10*).

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## Gastric Secretagogue Effect of Lysine Monohydrochloride

There have been recent references in medical literature to the use of lysine monohydrochloride as a nutritional supplement (*1*). Certain clinical responses, such as increase of appetite, weight gain, and rapid restoration of hemoglobin values, have been reported. Such effects might be produced by either or both of these mechanisms: (i) correction of pre-