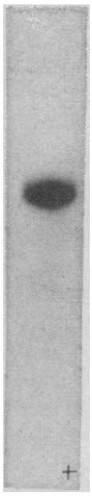

Fig. 2. Electrophoretic pattern of purified glycoprotein. Samples were treated with 1 percent sodium dodecyl sulfate and 0.01*M* phosphate buffer and subjected to electrophoresis on either 5 or 10 percent acrylamide gels in 0.1 percent SDS and 0.1*M* phosphate buffer, *p*H 7.0 (*15*). Duplicate gels stained with Coomassie blue for protein or periodic acid–Schiff reagent for carbohydrate show a single major component.

phenol in water. The mixture was stirred at 4°C for 15 minutes and centrifuged at 4000*g* for 1 hour at 4°C in a swinging bucket rotor. The centrifuged material separated into two phases; the upper (aqueous) phase contained most of the soluble glycoprotein. This was removed and dialyzed against several changes of distilled water at 4°C over a period of 24 to 36 hours. The dialyzed material, which sometimes was slightly turbid, was freeze-dried. The dry material was suspended in cold 100 percent ethanol and mixed for 1 to 2 hours in the cold and centrifuged to collect the precipitate. The ethanol washing procedure was repeated three times. The washed sediment was suspended in distilled water and dialyzed against water in the cold overnight. The material was then centrifuged at 10,000*g* for 30 minutes at 4°C; the clear supernatant contained the soluble glycoprotein.

The glycoprotein solution obtained by this procedure may contain a small amount of contaminating protein that is not removed during the phenol partitioning step. These contaminants can be removed by acidifying the solution with citric acid to *p*H 3.5 and then passing it through a phosphocellulose column equilibrated with 0.02*M* sodium citrate, *p*H 3.6. The glycoproteins do not bind to the cellulose under these conditions and emerge as a single peak.

Approximately 35 to 50 mg of glycoprotein can be extracted from 450 ml of human blood. This amounts to 3 to 4 percent of the original dry membranes and represents at least 70 to 80 percent of the total glycoprotein of the red cell membrane.

The glycoprotein prepared by this procedure appears to be a single molecular species, as judged by acrylamide gel electrophoresis (Fig. 2) in two different systems and by analysis of tryptic and cyanogen bromide peptides and COOH-terminal analysis (*9*). The monomeric unit has an apparent molecular weight of 55,000 (*10*), and the purified glycoprotein has A, B, and MN blood group activities and also carries the receptors for influenza viruses, phytohemagglutinin, and the wheat germ agglutinin (*11*). These activities were measured by hemagglutination-inhibition assays and by precipitin reactions with purified lectins (*12*).

These activities can also be demonstrated after the purified glycoprotein is extracted with a mixture of chloroform and methanol (2 : 1, at room temperature). This treatment removes contaminating lipids and eliminates the possibility that the blood group activities are due to glycolipids. The glycoprotein is still completely soluble in water after this treatment.

The above-described procedure can be used for the isolation of glycoproteins from other membranes. It has been used to extract proteins from lymphoid cells, platelets, liver cell membranes, and various human tumors, and there is evidence that it is effective for isolating tumor-specific antigens from human colonic tumors (*13*).

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7. LIS has a molar extinction coefficient of approximately 4×10^3 at 323 nm, and the presence of small amounts can be detected spectrophotometrically. The glycoprotein prepared according to this procedure has no detectable bound LIS.
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3 September 1971

Diethyl Pyrocarbonate: Formation of Urethan in Treated Beverages

Abstract. *Isotope dilution analyses with tritium-labeled diethyl pyrocarbonate show that the carcinogen urethan is formed in orange juice, white wine, and beer. Commercial use of the antimicrobial food additive diethyl pyrocarbonate can result in urethan concentrations of 0.1 to 0.2 milligram per liter in orange juice and of the order of 1 milligram per liter in white wine and beer.*

Diethyl pyrocarbonate (DEP),

$$H_5C_2\text{–O–CO–O–CO–O–}C_2H_5$$

also known as Baycovin, is a widely used antimicrobial food additive for beverages such as fruit juices, wine, and beer (*1, 2*). Diethyl pyrocarbonate hydrolyzes rapidly in water to form ethanol and carbon dioxide, but in the presence of suitable compounds it also reacts to some extent with these compounds forming carbethoxy derivatives and ethyl esters (*1, 3*). The hydrolyses and other reactions are completed within less than 24 hours after the addition of DEP to water, aqueous solutions, or beverages (*1, 2, 4*). A number of investigations on the formation of carb-

ethoxy derivatives in foods and with food components treated with DEP have been published (*1, 2, 4–8*), and some of these studies include toxicological evaluations (*5, 6*). A joint Food and Agriculture Organization–World Health Organization (FAO-WHO) committee on food additives has suggested that the use of DEP should be limited to beverages with a *p*H below 4.5 and with a low content of amino acids and proteins, and that the amount of DEP added should not exceed 300 parts per million (ppm) (*9*). Some authorities allow a maximum DEP content of 500 ppm (*2*).

Diethyl pyrocarbonate is known to react with ammonia in aqueous solution in the *p*H range from 4 to 9 forming urethan (ethyl carbamate) (*10*). The carcinogenic property of urethan has been known since 1943 (*11*). The joint FAO-WHO committee on food additives stated (*9*): "In the reaction products resulting from treatment with diethyl pyrocarbonate, analytical studies did not reveal the presence of ethyl urethan," citing one unpublished report from the manufacturer and one published report (*6*) in which, however, there is no mention of any study on urethan formation. Pauli and Genth (*1*) also quote an unpublished report indicating that the urethan concentration would be $\ll 0.01$ mg/liter in beverages treated with DEP.

In view of this inadequate information on urethan concentrations in DEP-treated beverages, we have performed some isotope dilution analyses with three common beverages: an orange juice, a beer, and a white wine (*12*). Tritium-labeled DEP (*13*) was added to 100-ml portions of the beverages at a refrigerator temperature of 4° to 8°C. The samples were stored at this temperature for at least 80 hours, after which 3.00 g of carrier urethan was added to each sample. The sample was then shaken with successive samples (100 to 150 ml each) of diethyl ether. The combined ether extract was evaporated, and the residue was dissolved in ethanol. The ethanol was distilled off at room temperature and reduced pressure. The residue containing crude urethan was recrystallized in petroleum ether (boiling point, 60° to 71°C). The process of dissolution in ethanol, evaporation of ethanol, and recrystallization in petroleum ether was repeated until the urethan had a constant specific activity. We measured the specific activities of the purified urethan samples as well as that of the DEP used in a scintillation counter, using a toluene solution of PPO (2,5-diphenyloxazole) plus POPOP {1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene}. The yield of urethan in the treated beverages was then calculated in a conventional manner. The background radioactivity was about 50 count/min, and 1 μl of DEP (1.12 mg) gave a count of about 174,000 count/min. The sensitivity of the analyses presented here can be exemplified by the fact that a radioactivity of 50 count/min above the background in a 100-mg urethan sample would correspond to a urethan yield of about 0.05 mg/liter in the beverage.

Table 1. Urethan yields in beverages treated with diethyl pyrocarbonate at 4° to 8°C.

Beverage	*p*H	Ammonium concentration (*19*) (mg/liter)	Amount of added DEP (μl/liter)	Urethan yield (mg/liter)
		Sample 1		
Orange juice	3.8	20	250	0.18
Orange juice	4.0*	20	250	.17
Orange juice	4.5*	20	250	.20
Orange juice	5.0*	20	250	.31
		Sample 2		
Orange juice	4.5*	20	250	.23
Orange juice	4.5*	20	500	.46
Orange juice	4.5*	20	1000	.58
		Sample 3		
Orange juice	4.5*	20	250	.21‡
Orange juice	4.5*	70†	250	.36
White wine	3.4	5	500	2.6‡
Beer	4.4	2	500	1.3‡

* The *p*H increased from 3.8 as a result of the addition of sodium hydroxide prior to the addition of DEP. † The ammonium concentration increased by 50 mg/liter as a result of the addition of diammonium citrate. ‡ Average of duplicate samples which deviated less than ± 20 percent from the average.

The analytical results and some data about the beverages are summarized in Table 1. The addition of 250 μl/liter of DEP to the orange juice resulted in the formation of about 0.2 mg of urethan per liter. The yield of urethan in the orange juice varies with the *p*H, the concentration of ammonium ion, and the amount of added DEP. The increase in the urethan yield in orange juice from *p*H 4 to 5, by about a factor of 2, is in agreement with our results obtained with ammonia and DEP in aqueous solutions (*10*). Whereas the urethan yield increases linearly when the amount of DEP added is increased from 250 to 500 μl/liter, this is not the case at still higher additions. The results at higher additions might be due to the fact that droplets of DEP remain undissolved in the liquid for an appreciable time after the addition of such large amounts of DEP. The chance for hydrolysis during dissolution is then greater and might result in a decreased effective concentration of DEP. The nonlinear relation between the ammonium ion concentration and the urethan yield is not unreasonable; such an effect has been noted for other compounds reacting with DEP in aqueous solutions (*8*). The reproducibility of the analyses can be evaluated by comparing the results for the three identical samples of orange juice treated at a *p*H of 4.5 with 250 μl of DEP per liter; the deviation from the mean value is less than 10 percent.

The urethan yields of 1 to 3 mg/liter in white wine and beer after an addition of 500 μl of DEP per liter are appreciably higher than that in orange juice. The yield of urethan as well as the yields of other derivatives are undoubtedly dependent on the composition of the beverage; it is thus also conceivable that different varieties of a particular beverage type might give rise to different urethan yields.

Ammonia is ubiquitous in plant and animal tissues (*14*). Citrus juices have been reported to contain up to 50 mg/liter (*15*). Analyses of wines and musts have revealed ammonia concentrations between 5 and 128 mg/liter (*16*). Beer also contains ammonia (*17*).

It is thus very probable that foods treated with DEP invariably will contain the carcinogen urethan. It has been pointed out (*18*): "What zero

tolerance in respect of food additives really means is that deliberate addition to the carcinogenic burden already upon us should be avoided where this is at all feasible."

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13. The ^{3}H-labeled DEP (supplied through the courtesy of Dr. B. Öberg) was diluted 500 times with inactive DEP; the result was a sample with a specific activity of 22 μc/mmole which was used in the investigation reported here. The synthesis of the ^{3}H-labeled DEP is described in: B. Öberg, *Eur. J. Biochem.* **19**, 496 (1971).
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26 July 1971; revised 25 August 1971

Single Neuron Activity in Cat Gigantocellular Tegmental Field: Selectivity of Discharge in Desynchronized Sleep

Abstract. *Ratios of discharge rates in desynchronized sleep to those in waking and synchronized sleep of gigantocellular neurons are five to ten times higher than are those of neurons in adjacent tegmental fields and 25 to 30 times higher than in other brain sites. This marked concentration of activity in desynchronized sleep is compatible with an active role of gigantocellular neurons in the generation of this sleep phase.*

Evidence from lesion and stimulation studies implicates pontine brainstem structures as important in the generation of desynchronized sleep (D) (*1*). However, boundaries of the critical region (or regions) and identity of the controlling neural elements remain vague because, apart from being unphysiological, lesions and stimulation can produce their effects through action on fibers arising elsewhere as well as through action on somata intrinsic to the area studied. More precise and more physiological information about the locus and nature of neural elements generating activity characteristic of this phase of sleep can be sought through recordings of single cells.

Neurons that are a part of a central system actively controlling D sleep should show increases in discharge rates during this behavioral state which are greater than those of presumed "follower" neurons, such as those in cerebral and cerebellar cortices (*2*). We thus suggest that the degree to which activity is concentrated in D sleep, or the *selectivity* of firing in this state can be taken as one important criterion for testing the hypothesis that a given group of cells is at or near the origin of the increased neuronal activity during D sleep. The selectivity of discharge during D can be quantified through the ratio of D discharge rates to those of waking (W) and synchronized sleep (S).

The large neurons of the pontine reticular formation in the gigantocellular tegmental fields (FTG) are, on the basis of anatomical considerations, possible candidates for this controlling role. Brodal (*3*) has presented evidence that more than one-half of the 3000 to 4000 giant cells in the cat FTG send ascending axons beyond midbrain, and more than one-half send descending axons into spinal cord. Included in these estimates are those cells that have both ascending and descending axon branches. These direct and widespread connections to regions outside the brainstem suggest that these cells could effect the ubiquitous and marked changes in brain and spinal neuronal activity that occur during D sleep. The FTG corresponds to the nucleus reticularis pontis oralis *and* nucleus reticularis pontis caudalis, structures that the studies cited above (*1*) have implicated in the control of D sleep.

In our study we use previous observations (*2*) of the selectivity of discharge of cerebral and cerebellar cortical neurons and we analyze new measurements of the discharge rates of neurons in several brainstem sites. The results are compatible, in terms of the selectivity criterion, with a controlling role for FTG neurons in the generation of events during D sleep.

Under pentobarbital anesthesia, four cats were implanted with electrodes for recording electrographic data [electrooculogram (EOG), parietal electroencephalogram (EEG), transcortical EEG, and nuchal electromyogram (EMG)] by methods previously described (*4*). In addition, a steel plate was fixed to the frontal sinus; this plate had threaded bolts extending from it that were screwed to a metal bar at the time of recording; this was done to minimize the head-on-neck movements that are a major source of instability in brainstem unit recordings. Extracellular single unit activity was recorded from glass-insulated platinum-iridium microelectrodes that were controlled by an Evarts-type micromanipulator (*5*). The cylinder for holding the micromanipulator was positioned over the vermian cortex of the cerebellum and mounted on the bone at an angle of 30° from the vertical. The anterior-posterior position of the cylinder center was varied to give a window on the brainstem that included, rostrally, the upper pons and, caudally, the upper medulla. The